National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

The 25th EURL-*Salmonella* workshop

17 and 18 September 2020, Online

RIVM report 2020-0202
K.A. Mooijman

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Colophon

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Synopsis

The 25th EURL-*Salmonella* workshop
17 and 18 September 2020, Online

This report gives a summary of the presentations held at the 25th annual workshop for the European National Reference Laboratories (NRLs) for *Salmonella* (17-18 September 2020). The aim of the workshop was to facilitate the exchange of information on the activities of the NRLs and the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*). The workshop was organised as an online meeting for the first time due to the SARS-CoV-2-virus pandemic.

Annual Proficiency Tests
A recurring item at the workshops is the presentation of the results of the annual Proficiency Tests organised by the EURL. These provide information on the quality of the participating NRLs. In 2020, for the first time a Proficiency Test for the detection of *Salmonella* in mussels was organised. The NRLs had high scores in the 2019-2020 studies; detailed information on the results per Proficiency Test is available in separate RIVM reports.

Next Generation Sequencing
Whole Genome Sequencing is nowadays the method of choice for sub-typing microorganisms. In several presentations the application of this sequencing technique for *Salmonella* was shown. Especially for outbreak investigations this technique has shown to be a valuable tool.

Analytical methods
In the last session of the workshop, presentations were given on analytical methods for detection and typing of *Salmonella*. Also the procedures for validation and verification of methods were explained.

The workshop was organised by the EURL-*Salmonella*, part of the Dutch National Institute for Public Health and the Environment. The main task of the EURL-*Salmonella* is to evaluate the performance of the European NRLs in detecting and typing *Salmonella* in different products.

Keywords: EURL-*Salmonella*, NRL-*Salmonella*, *Salmonella*, workshop 2020

Publiekssamenvatting

De 25e EURL-*Salmonella* workshop
17 en 18 september 2020, Online

Het RIVM heeft de verslagen gebundeld van de presentaties van de 25e workshop voor de Europese Nationale Referentie Laboratoria (NRL's) voor *Salmonella* (17-18 september 2020). Deze workshop wordt elk jaar georganiseerd. Het doel is dat het overkoepelende orgaan, het Europese Referentie Laboratorium (EURL) voor *Salmonella*, en de NRL's informatie uitwisselen. Door de uitbraak van het coronavirus is de workshop online georganiseerd.

In elke workshop is er veel aandacht voor de ringonderzoeken die het EURL elk jaar organiseert om de kwaliteit van de NRL's te controleren. In 2020 is voor het eerst een ringonderzoek georganiseerd om *Salmonella* in mosselen te analyseren. De NRL's scoorden goed in de ringonderzoeken van 2019 en 2020. In dit rapport staan de ringonderzoeken kort beschreven. Uitgebreide informatie staat in de rapporten die over elk ringonderzoek worden uitgegeven.

Om *Salmonella* heel precies te karakteriseren wordt Whole Genome Sequencing gebruikt. Verschillende presentaties lieten zien dat deze techniek goed te gebruiken is voor *Salmonella*. Vooral bij onderzoek naar uitbraken is deze techniek zeer waardevol.

Andere presentaties gaven informatie over andere methoden om *Salmonella* aan te tonen en te karakteriseren. Ook zijn de procedures om methoden te valideren en verifiëren uitgelegd.

Het EURL voor *Salmonella*, dat onderdeel is van het RIVM, organiseert deze workshop. Een belangrijke taak van het EURL-*Salmonella* is de kwaliteit van de nationale referentielaboratoria voor deze bacterie in Europa controleren.

Kernwoorden: EURL-*Salmonella*, NRL-*Salmonella*, *Salmonella*, workshop 2020

Contents

Summary

On 17 and 18 September 2020, the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) organised its annual workshop. Due to the SARS-CoV-2-virus pandemic it was not possible to organise a physical meeting. Hence, for the first time in the history of the EURL-*Salmonella* the workshop was organised as a virtual meeting. Participants in the workshop were representatives of the National Reference Laboratories (NRLs) for *Salmonella* from 27 European Union (EU) Member States, three European Free Trade Association (EFTA) countries, and five (potential) EU candidate countries. Also present were representatives of the European Commission Directorate-General for Health and Food Safety (EC DG SANTE) and of the European Food Safety Authority (EFSA). A representative of the NRL-*Salmonella* of one EU Member State was unable to join the workshop. Thanks to the fact that this workshop was organised as a virtual meeting, it was possible to host more participants than before. In total 75 participants attended.

During the workshop, presentations were given on several topics. In the first session, the results of the Proficiency Tests (PTs) organised by the EURL-*Salmonella* in the past year were presented, namely the PT on detection of *Salmonella* in chicken faeces samples (October 2019), the PT on detection of *Salmonella* in mussels (organised in 2 rounds: March and August 2020), and the PT on *Salmonella* typing (November 2019).

The second session focused especially on Next Generation Sequencing (NGS). Presentations were given on cluster analysis, on the European Commission mandate on 'One Health' system for collection and analysis of Whole Genome Sequencing (WGS) data from food/animal isolates, on a *Salmonella* Enteritidis outbreak in a hotel school in Belgium, and on WGS comparison of multi drug resistant *Salmonella* Infantis isolates from broilers and humans in the Netherlands.

In the third and last session, presentations related to analytical methods were given. These concerned presentations on development and testing of draft ISO/TS 6579-4 (on identification of monophasic *Salmonella* Typhimurium), on comparison of *Salmonella* Typhimurium and monophasic variants in East Anglia and on verification of methods following EN ISO 16140-3; theory and practice.

The workshop concluded with a presentation on the EURL-*Salmonella* work programme for the current and coming year.

The workshop presentations can be found at the website of the EURL-*Salmonella*: https://www.eurlsalmonella.eu/workshops

1 Introduction

This report includes the abstracts of the presentations given at the 2020 EURL-*Salmonella* workshop, as well as a summary of the discussion that followed the presentations. The full presentations are not included in this report, but are available on the EURL-*Salmonella* website (subject to publication permission): https://www.eurlsalmonella.eu/workshops

The layout of the report is consistent with the workshop programme.
Chapter 2 includes the abstracts of the presentations given on the first day.
Chapter 3 includes the abstracts of the presentations given on the second day.
The workshop is evaluated in Chapter 4; the evaluation form template can be found in Annex 3.
The list of participants is given in Annex 1.
The workshop programme is given in Annex 2.

2 Thursday 17 September 2020: Day 1 of the workshop

2.1 Opening and introduction

Kirsten Mooijman, Head of EURL-Salmonella, Bilthoven, the Netherlands

Kirsten Mooijman, head of the European Union Reference Laboratory (EURL) for *Salmonella*, opened the 25th workshop of the EURL-*Salmonella*, welcoming all participants to this first virtual workshop of the EURL-*Salmonella*.

In total, 75 participants attended this workshop, including representatives of the National Reference Laboratories (NRLs) for *Salmonella* from 27 EU Member States, five (potential) candidate EU countries, and three member countries of the European Free Trade Association (EFTA). Additionally, representatives of the European Commission Directorate-General for Health and Food Safety (EC DG SANTE) and of the European Food Safety Authority (EFSA) attended the workshop. Apologies were received from the representative of the NRL-*Salmonella* in Malta.

The evaluations of the last nine workshops (2011–2019) were compared. The opinion on the scientific programme was the same in all workshops: very good to excellent.

The workshop started after the presentation of the programme and general information. The workshop programme can be found in Annex 2.

2.2 Results EURL-*Salmonella* Proficiency Test Primary Production 2019 – Detection of *Salmonella* in chicken faeces samples

Irene Pol-Hofstad, EURL-Salmonella, Bilthoven, the Netherlands

In October 2019, the annual EURL-*Salmonella* Proficiency Test (PT) on detection of *Salmonella* in primary production stage samples was organised. A total of 35 NRLs participated in this study: 29 NRLs originating from 28 EU-Member States (MS), five from third European countries (EU candidate or potential EU candidate MS and members of the EFTA), and one from a non-European country. Participation was obligatory for all EU Member State NRLs responsible for the detection of *Salmonella* in primary production stage samples.

Chicken faeces from a pathogen free farm was used in this study. The chicken faeces samples were artificially contaminated with a diluted culture of *Salmonella* Typhimurium at the EURL laboratory.

Each NRL received sixteen blindly coded samples consisting of ten chicken faeces samples artificially contaminated with two different levels of *Salmonella* Typhimurium: six low (MPN concentration: 13 cfu/sample), and four high contaminated samples (MPN concentration: 35 cfu/sample). Additionally, four negative chicken faeces samples (no *Salmonella* added) and two control samples had to be analysed. The control samples consisted of a procedure control blank and a control sample to be inoculated by the participants using their own positive control strain. The

samples were stored at 5 °C until the day of transport. On Monday 23 September 2019, the contaminated chicken faeces samples were packed and sent to the NRLs. On arrival, the NRLs were asked to store the samples at 5 °C until the start of the analysis.

Most laboratories used the prescribed method EN ISO 6579-1:2017, one laboratory used EN ISO 6579:20072/Amd.1:2007 (Annex D), and one laboratory used another method.

All but one of the laboratories scored well, analysing both the procedure control as well as their own positive control sample correctly. One laboratory mislabelled the control samples. This laboratory scored a moderate performance.

All laboratories detected *Salmonella* in the chicken faeces samples contaminated with a low level of *Salmonella*. One laboratory found one of the six samples negative for *Salmonella*, another laboratory found two of the six samples negative for *Salmonella*. These results are still within the criteria for good performance, which permit three negative samples.

All but one of the laboratories detected *Salmonella* in all four high level samples. One laboratory scored one of the four high-level samples negative. This is still within the criteria for good performance which permit one negative sample. The sensitivity score was 98,6% for these samples.

All negative samples were scored correctly negative, resulting in a specificity of 100%.

Overall, the laboratories scored well in this Proficiency Test with an accuracy of 99,2%. Thirty-four laboratories fulfilled the criteria of good performance. The results of one laboratory were scored moderate due to a labelling error.

More details can be found in the report of this PT (Pol-Hofstad and Mooijman, 2020).

Discussion
Q: Is the planned PT the one for primary production of shellfish?
A: No, the PT of March (and August) 2020 concerned the detection of *Salmonella* in shellfish. This planned PT is the combined PPS-Food PT (not shellfish) planned in September/October 2020.

2.3 Preliminary results EURL-*Salmonella* Proficiency Test Live Bivalve Molluscs 2020 – Detection of *Salmonella* in mussels

Robin Diddens, EURL-Salmonella, Bilthoven, the Netherlands

In March 2020, an EURL-*Salmonella* PT on detection of *Salmonella* in Live Bivalve Molluscs (LBM) was organised for the NRLs-*Salmonella.* The matrix to be analysed was mussels. Due to preventative measures related to the SARS-CoV-2-virus pandemic, not all NRLs were able to participate in March. Therefore, a second round of this PT was organised in August 2020 in which nine NRLs participated.

In total, 23 NRLs-*Salmonella* participated in this PT: 20 NRLs from 20 EU Member States (MS) and three NRLs from third countries (EU candidate MS and members of the EFTA).

Reference materials were used for spiking the mussel samples. Prior to the PT, pre-tests were conducted to make sure that the mussels and the reference materials were fit for use. Two *Salmonella* serovars and different inoculation concentrations were tested. In addition, the concentration of the natural background flora in the mussels (aerobic count and *Enterobacteriaceae)* was tested. Based on these results, custom made *Salmonella* Typhimurium (STm) reference materials were ordered with a concentration of approx. 100 cfu STm/ml. The aim was to spike the mussels with approx. 10 cfu STm/sample, so 100 µl reference material per sample had to be used.

Each participant received two parcels. One parcel containing four vials of (*Salmonella*) reference materials, packed in dry-ice. The second parcel contained a 2 kg package of mussels (Modified Atmosphere Packaging), including cooling blocks and an electronic temperature device.
Following a protocol, each NRL had to prepare four samples of each 25 g of mussel flesh and intravalvular fluid. Next, the laboratories had to spike each sample with 100 µl of the reference material with the corresponding sample number. Three reference materials contained *Salmonella* Typhimurium (vials A, B and D) and one reference material did not contain *Salmonella* (vial C).
The NRLs also had to test two control samples in the PT: a procedure control (only Buffered Peptone Water) and a positive control with *Salmonella*.

The concentration of *Salmonella* in the reference materials was tested at the EURL-*Salmonella* at several moments between February and July 2020, after storage at -70 °C. The concentration varied between $1{,}2 \times 10^2$ cfu STm/ml and $1{,}3 \times 10^2$ cfu STm/ml. The inoculation levels of *Salmonella* Typhimurium in the mussel samples were tested at the EURL-*Salmonella* at the start of the PTs, and were 13 cfu/mussel sample in March and 12 cfu/mussel sample in August 2020. The background flora in the mussels was also tested in March and August. In March 2020, the concentration of *Enterobacteriaceae* was <10 cfu/g and the aerobic count was $7{,}3 \times 10^3$ cfu/g. In the batch of mussels used in the second round of the PT in August, the concentration of *Enterobacteriaceae* was $2{,}5 \times 10^2$ cfu/g and the aerobic count was $9{,}2 \times 10^2$ cfu/g.

Twenty-one laboratories fulfilled the criteria of good performance in this PT for the detection of *Salmonella* in mussel samples. One laboratory scored a moderate performance, as this NRL mixed up the control samples and therefore also reported the results in the wrong order. One Laboratory scored an unsatisfactory performance because it detected *Salmonella* in sample C (negative sample). The NRL was asked for a technical explanation of their deviating results. The serotyping result of sample C was *S.* Typhimurium, which was the same serovar used in the reference materials. The remaining reference materials as well as the mussel samples were kept frozen at the NRL and were tested again. In both vial C and the mussel sample C, *Salmonella* was not detected. Cross-contamination was probably the reason for the initial deviating

result. For this NRL, a follow-up study was organised in August 2020 (at the same time as the second round of the PT was scheduled). For the follow-up study, the same batch of mussels was used as in the second round of the PT. Again four vials of *Salmonella* reference materials were sent to the NRL. This time two vials contained *Salmonella* Typhimurium and two vials did not contain *Salmonella*. The laboratory was asked to spike the mussel samples with a higher amount of reference material (500 µl) than in the full PT (100 µl). This would result in an inoculum of 55 cfu STm/sample. The NRL scored a good performance in the follow-up study.

More details can be found in the interim summary of this PT (Diddens and Mooijman, 2020).

Discussion
Q: Is the low number of test samples justifiable?
A: From a statistical point of view, this number of samples may be low, but this was the best we could do from a practical point of view. Still it was possible to detect 'big mistakes' in this PT. This was our first PT for detection of *Salmonella* in shellfish. We may consider to use another set-up in a next PT, so that perhaps more samples can be tested (e.g. spiking of mussel flesh instead of using whole live mussels). *Note: the former EURL for live bivalve molluscs only distributed an average of three samples per PT for detection of* Salmonella *in live bivalve molluscs, due to practical limitations for this type of sample.*

2.4 Results EURL-*Salmonella* Proficiency Test Typing 2019 - serotyping and cluster analysis

Wilma Jacobs-Reitsma, EURL-Salmonella, Bilthoven, the Netherlands

In November 2019, the 24th PT on typing of *Salmonella* was organised by the EURL-*Salmonella*. A total of 35 laboratories participated in this PT, consisting of an obligatory serotyping part and an optional part on cluster analysis, the latter being a pilot study. Participants included 29 NRLs-*Salmonella* of the 28 EU Member States, 3 NRLs of EU-candidate countries, and 3 NRLs of EFTA countries. The main objective of this PT was to check the performance of the NRLs for serotyping *Salmonella* spp. and to compare the results of serotyping *Salmonella* spp. among the NRLs-*Salmonella*.

A total of 20 obligatory *Salmonella* strains plus 1 additional *Salmonella* strain from an uncommon type were selected for the serotyping part of the PT by the EURL-*Salmonella*. The strains had to be typed with the method routinely used in each laboratory, following the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007).

The individual laboratory results on serotyping, as well as an interim summary report on the general outcome, were emailed to the participants in February 2020.

The O-antigens were typed correctly by 32 of the 35 participants (91%). This corresponds to 99% of the total number of strains. The H-antigens were typed correctly by 27 of the 35 participants (77%), corresponding

to 97% of the total number of strains. As a result, 26 participants (74%) gave the correct serovar names to the full set of strains, corresponding to 97% of all strains evaluated. A completely correct identification was obtained for seven *Salmonella* serovars: Poona (S3), Enteritidis (S5), Montevideo (S13), Virchow (S16), Infantis (S17), Saphra (S18), and Kingston (S20). Another eight serovars were completely correctly named after excluding the results of a newly participating laboratory, with a relatively high number of mistakes. Five participants did not have access to the required but less common antisera to completely name strain S4 (*S*. Fresno, 9,46:z_{38}:-). All but five participants tried to serotype optional strain S21, a *Salmonella enterica* subsp. *houtenae* (IV). A number of laboratories did not have access to the required antisera to finalise this (48:g,z_{51}:-).

Overall, the performance of the NRLs in the PT Serotyping 2019 was very good. Two participants (non-EU MS) did not meet the level of good performance at the first stage of the study. The follow-up on this by the first participant was satisfactory, but the proposed training session for the second participant had to be put on hold due to the ongoing SARS-CoV-2-virus pandemic.

The individual laboratory results on the cluster analysis part and an interim summary report on the general outcome were emailed to the 18 participants in June 2020.

The participants were asked to investigate a set of 10 *Salmonella* strains. Participants could choose how they performed cluster analysis, by PFGE and/or MLVA and/or WGS (or any combination of these methods), and using their own routine method(s) of choice.
A total of 18 NRLs participated in the cluster analysis pilot, with 6 participants for PFGE analysis, 8 for MLVA analysis and 14 participants for WGS analysis. Details on the method(s) used and the outcome of the cluster analysis had to be reported in the electronic result form. Additionally, specific data for PFGE and WGS had to be sent by e-mail or to be uploaded to a secure ftp server.

This first pilot study on analysis clusters led to a large amount of interesting information and data. The participants were free to use their own interpretation of ‘cluster(s) of closely related isolates’. Therefore, no performance criteria were set for this pilot study. As a minimum, it was expected that the participants would report the technical duplicate strains SCA03 and SCA06 to be (part of) one cluster. This was found in 5/6 (PGFE), 8/8 (MLVA), and 12/14 (WGS) of the cases. Cluster definition however, was interpreted in a variety of ways, which made an appropriate evaluation more complex.

Additional investigations at the EURL-*Salmonella* are ongoing, aiming to clarify the observations on the variability of the WGS results on some of the strains, and to support the selection of suitable PT strains.

With the lessons learned from the first pilot in 2019, a second pilot study on cluster analysis will be organised in 2020.

More details can be found in the interim summaries of this PT (Jacobs-Reitsma et al., 2020a, b).

Discussion
Q: Does the assembly-pipeline for WGS contain any assembly correction step e.g., the Pilon software?
A: No, we plan to improve the current pipeline at the RIVM.
Q: I'm not sure if Ridom has included an assembly correction. It would be interesting to have the exact versions and the complete pipeline for assembly for at least the cgMLST analysis.
A: This information will be given in the report of the PT Typing 2019.
Q: The tree presented was done starting from the fastq of the participants or from assembly?
A: It was started with the fastq files of the participants using an in-house pipeline that uses SPAdes 3.10.0 to obtain assemblies. These assemblies generated the tree presented.

2.5 Cluster analysis of WGS-data

Joakim Skarin, EURL-Campylobacter, Uppsala, Sweden

A 'Guidance document for cluster analysis of WGS data' has been produced in the context of the Inter-EURLs working group on next generation sequencing (inter EURLs WG on NGS). It informs and supports NRLs in their choices of methods to be used for the so-called cluster analysis, in which comparisons of genomes are performed followed by visualisations of the results to allow an interpretation of how closely the genomes are related. This type of analysis can be used in outbreak investigations where the source of an outbreak is determined. The routes of infection and the spread of an outbreak clone can be analysed to create interventions to stop future outbreaks.

Broadly, the most common comparison approaches can be divided into (i) the single nucleotide polymorphism (SNP) approach where individual mutations are used as separate phylogenetic markers, and (ii) the gene-by-gene approach, where each variant of a gene is considered a phylogenetic marker.
The guidance document highlights differences between the two approaches and the steps that need to be carefully validated to be able to perform a cluster analysis. The document lists the software available for SNP and gene-by-gene methods, both for local and for online operation.
The SNP-based methods represent the highest resolution in relatedness studies and the gene-by-gene approaches represent a more easily standardised way of analysis that enables sharing of comparable cluster analysis results. Both methods can produce comparable results when applied to outbreak investigations, and any differences are discussed in the document.

The guidance document for cluster analysis of WGS data is available through the websites of the EURLs which are members of the inter-EURLs WG on NGS. For example, see the NGS page at https://www.eurlsalmonella.eu/publications/eurl-manual.

Discussion
Q: When will the guidance documents of the joint EURLs working group on NGS become available?
A: It is planned to publish these guidance documents at the EURLs' websites before the end of 2020.
Q: Can you spend a few words on reference sequence selection?
A: This may depend on what you want to do with reference sequences. It may be of interest to have reference sequences for the genotype you are looking for. For example, if you look for *Salmonella* Dublin in an outbreak, it is nice to also have this serovar as a reference sequence. If you want to create a cgMLST scheme, you may need a large validation set of genomes to cover, for example, the variability of the whole species, if the cgMLST scheme covers a species.

2.6 European Commission mandate on 'One Health' system for the collection and analysis of whole-genome sequencing (WGS) data from food/animal isolates

Mirko Rossi, EFSA, Parma, Italy

As follow-up to the ECDC EFSA technical report focusing on evaluating possible solutions for collecting and analysing WGS data (ECDC et al., 2019), the European Commission Directorate-General for health and food safety (EC DG SANTE) issued a request to ECDC and EFSA for the implementation of 'One Health system for the collection and analysis of whole-genome sequencing (WGS) data from human and food/animal isolates' (M-2020-0015).

The 'One Health' system will support signal detection of multi-country events based on cgMLST, and it will be composed of two interoperating systems, hosting two databases: one for human data received by ECDC and one for non-human data received by EFSA. Each system is based on a workflow which collects and stores the data (i.e. allelic profiles and descriptive data) of the respective data domain. Each time there is the need to detect cross-sector matches, the two databases will interact programmatically by exchanging cgMLST allelic profiles and limited descriptive data as established in a Collaboration Agreement, allowing joint signal detection in real-time. To avoid the use of a centralized nomenclature server, the hash function of the nucleotide sequence of the detected allele will be used as allele designation. Once a cluster of human and non-human allelic profiles is detected and further investigation is agreed to be necessary, additional WGS data can be exchanged between the agencies either through the databases or in an ad-hoc manner.

This separation and the exchange of, in first instance, only the profiles of encrypted alleles between EFSA and ECDC will allow the agencies to develop the system independently from each other, taking in consideration the different needs and constraints of respective data providers. The final aim is to increase provision of data from EU Member States (MSs), especially regarding Food and Veterinary data, and to improve data sharing particularly by providing solutions that guarantee a greater level of protection of the data.

The use of profiles of encrypted alleles along with the descriptive data will allow EFSA to collect the allelic profile from MS rather than raw WGS data only, decentralizing the computing resources needed. As such, raw data can remain at the owner level, only the results are shared.

MSs will interact with the relevant database per sector in their role of data providers, by submitting raw sequencing data or processed data (depending on the sector and the preference of each country), and in their role of data users by accessing and querying the relevant database, performing data analysis and visualising the results. Both data provision and access will be managed through user-friendly interfaces, and according to predefined rules.

Discussion
Q: Is data that is logically deleted still connected to a specific isolate?
A: Yes, this will remain visible for the data provider, but other users will no longer see this entry.
Q: Will the data be open to institutions independent of whether they are classified as EU members or external?
A: The system is intended for EU member states to support EFSA, ECDC and EC in multi-country outbreak investigations. Only risk assessors and institutions recognised by a member state will be able to see the data, after agreement with EFSA. Whether UK institutions have access to the database may depend on what is agreed with the United Kingdom after leaving the EU.
Q: Is this system tailored towards analysis data obtained from current common sequencing technologies (e.g. Illumina MiSeq etc.) or can long-read data also be used (e.g. MinION data)?
A: The current focus of the system is on short reads. However, we will try to design the system to be sufficiently flexible to implement any type of changes when needed. We are designing the system to accept Illumina sequences and Ion Torrent sequences, but it is not always easy to merge the results generated from these two sequence technologies.
Q: Do you have any thoughts on the pros and cons of open access sequence storage?
A: My personal view is that open access data facilitate outbreak detection and international collaboration, but not all countries are ready or have the possibility for giving open access to their sequences. Therefore, it is important to foresee a close secure network, strongly regulated, open to a specific circle of trust.

2.7 *Salmonella* Enteritidis outbreak in a hotel school in Belgium

*Sarah Denayer, NRL-*Salmonella*, Brussels, Belgium*

In September 2019, a *Salmonella* outbreak occurred in a hotel school in Bruges, Belgium. Over 200 students and teachers were infected and the Belgian National Reference Centre (NRC) and National Reference Laboratory (NRL) rapidly received samples from human and food origin, respectively. The analysis consisted of isolation, serotyping, MLVA comparison, followed by whole genome sequencing (WGS) as currently recommended by EFSA for this pathogen. The source of the contamination was confirmed about two weeks after reception of the samples at the Reference Laboratory; freshly prepared tartar sauce in a

meal cooked at the school. The food isolates from the whole meal sample and the isolates of its components separately (tartar sauce) were confirmed to be identical to the strain detected in the patients. These outbreak strains were separated from sporadic cases as well as from another outbreak circulating in Europe in the same period. In parallel, the enriched cultures of the food samples were evaluated with a short-reads shotgun metagenomics approach. *Salmonella* was confirmed in both samples and an inferred genome of a *Salmonella enterica* subsp. *enterica* serovar Enteritidis could be linked to the human isolates of the outbreak in a phylogenetic tree.

Discussion

Q: On what sequencing platform did you perform the metagenomics?
A: The metagenomics analysis was performed on a MiSeq platform.
Q: Did you enrich for bacterial DNA from the matrix?
A: DNA was extracted from the enriched broth (ISO 6579). The method is described in Buytaers et al., 2020.
Q: What is the minimum threshold for the percentage of a pathogen compared to total reads in food to be indicative for the causative agent?
A: In this particular case, a specific enrichment for *Salmonella* was performed and thus it was most likely to detect the pathogen. This could be matched with the available epidemiological information (related to the disease symptoms) and the detection of *Salmonella* in a human case when the suspected food was still under analysis. qPCR screening revealed possible presence of *Salmonella* in the food item. Specific bioinformatics tools were used to confirm that we only had 1 pathogen, and that it was *Salmonella*. Indeed, bioinformatics tools might not all give similar results and this is something to be improved. In a study with STEC (Buytaers et al., 2020), we were able to detect endogenous *E.coli* from spiked STEC, or 2 different STEC strains spiked concomitantly. Taking this all into account, we should ideally be able to detect the pathogen(s) even if multiple strains of the same species are present on the sample, but currently only after enrichment.

In order to be sure that on detecting a read, it comes from a species present in the sample, some people are trying to determine a kind of level of detection (LOD). This needs to be done by verification with qPCR for all the different pathogens and in different set-ups (concentrations, presence other species etc). This should also be related to the disease, whether it fits with the symptoms, and whether it is the 'causative agent'. Additionally, the infectious dose may vary per pathogen and if the infection was caused by a virus, the methods will have to be adapted to also include those in a diagnostic approach. This is not an easy task. But hopefully, after more metagenomics studies have been performed, more information will become available.
Q: Do you know the concentration of *Salmonella* Enteritidis in the matrix?
A: The initial concentration of *Salmonella* in the matrix was not evaluated. We worked with enriched matrices.
Q: What would be the general cost to run one sample?
A: The cost per sample is highly dependent on the individual price agreement between Illumina and the institution and depends mainly on the capacity of the institution (number of analyses made per year), so no exact cost can be given. To give you an idea and enable your own

calculations, we did 8 samples in one MiSeq run, so the cost of an MiSeq cartridge (major consumable cost) needs to be divided by 8. If you analyse only 1 sample, the cost would at least be the cost of 1 cartridge. The costs will generally be more than €300/sample and will include the consumables for sequencing (i.e. 1 cartridge/8 samples and library prep per sample), but also other costs such as DNA extraction, maintenance, personnel, data analysis and others. Using another sequencing platform, will also change the cost per sample (and possibly also the depth of sequencing).

2.8 Whole genome sequence comparison of MDR *Salmonella* Infantis isolates from broilers and humans in the Netherlands

Angela van Hoek, EURL-Salmonella, Bilthoven, the Netherlands

In recent years, there has been a shift from *Salmonella* Paratyphi B var. Java to *S.* Infantis being the most prevalent *Salmonella* serovar in broilers and on broiler meat in the Netherlands as well as in Europe. Among human cases in the Netherlands and in Europe, *S*. Infantis is the fourth most reported serotype. This often concerns multidrug resistant (MDR) variants carrying a pESI-like megaplasmid, which can enhance biofilm formation and the attachment/invasion efficiency of this pathogen. Whole genome sequence (WGS) analysis was performed to investigate the occurrence and to compare *S*. Infantis with a pESI-like megaplasmid among broilers and human cases in the Netherlands.

In 2018-2019, faecal samples were collected from 194 broiler farms in the Netherlands and screened for the presence of *Salmonella*. The obtained isolates were analysed with an xMap *Salmonella* Serotyping kit (Biovet) in order to select for *S*. Infantis. *S*. Infantis isolated from broilers (n= 14), together with one isolate from a healthy broiler farmer and a selection of Dutch MDR clinical isolates from 2018-2019 (n= 12) obtained from national surveillance were analysed with short-read sequencing on Illumina platforms. Core genome MLST (cgMLST) analysis was performed to compare the isolates after *de novo* assembly. BLAST (basic local alignment search tool) analysis was used to screen for pESI-like plasmid linked genes as well as for antimicrobial resistance (AMR) genes.

Overall *Salmonella* was isolated from 23 farms (11,9%, 95%CI: 8,0-17,2%) with *S*. Paratyphi B var. Java (n=12) and *S*. Infantis (n=10) being the most prevalent serotypes. cgMLST analysis with nearly all of the 3002 alleles present in the *Salmonella* scheme included, demonstrated a considerable variance among the *S*. Infantis isolates analysed. Clusters were identified containing only human isolates, but also ones with a mix of broiler and human *S*. Infantis. BLAST analysis looking for the pESI-like plasmid revealed a high occurrence among the isolates included in this study. The prevalence among the MDR human isolates was 100% (95% CI: 79-100%), and 93% (95% CI: 69-99%) of the broiler isolates were positive for the megaplasmid. The minimum spanning tree based on cgMLST analysis showed a separation of isolates harbouring the megaplasmid and the negative ones. The overall number of AR genes among human isolates was larger than in *S*. Infantis from broilers. The ESBL encoding gene *bla*$_{\text{CTX-M-65}}$ was found among 42% of

the human isolates, while no ESBL-producers were identified in the broiler isolates.

In conclusion it was shown that *S*. Infantis isolates carrying the pESI-like MDR megaplasmid were present in Dutch broilers and among isolates from human cases. There was a large variance among the genomes of *S.* Infantis, but some broiler isolates did cluster together with human isolates, which might indicate that *S*. Infantis in Dutch broilers could act as one of the sources of human infections in the Netherlands.

Discussion
Q: Very interesting that you only found one *Salmonella* per farm and only two main serovars. You did not find *S.* Enteritidis?
A: We did not find *S.* Enteritidis in broilers. In the Netherlands *S.* Paratyphi B var. Java is commonly found.
Q: On the minimum spanning tree, there is one human isolate that is 8 alleles apart from the broiler isolate - why not set the threshold to 8 alleles?
A: The threshold was based on that used by EFSA and ECDC, and they often use a threshold of 5-7 alleles difference.
Q: What is the difference between farms and flocks in terms of the sampling?
A: At least 1 flock was sampled per farm. If a farmer had 2 broiler houses, if allowed, both flocks were sampled. Sometimes 2 isolates from 1 broiler house were analysed and these were indistinguishable.
Q: Has the ESBL pESI plasmid spread in recent years or has it been present for a longer time?
A: In recent decades, many people have reported this plasmid. Remark: In the UK, the plasmid has been present in humans for the past few years in small numbers but has been increasing every year. Still it is relatively low in the *S.* Infantis population in humans.

3 Friday 18 September 2020: Day 2 of the workshop

3.1 Development and testing of draft ISO/TS 6579-4: Identification of monophasic *Salmonella* Typhimurium

Robin Diddens, EURL-Salmonella, Bilthoven, the Netherlands

Regulatory limits (microbiological criteria) for *Salmonella* have been set out for food specified in Regulation (EC) No. 2073/2005, which lays down *Salmonella* food safety criteria. These prescribe that *Salmonella* spp. are 'not detected in 25 g or 10 g' of different products when on the market, throughout their shelf life. Moreover, according to Regulation (EC) No. 1086/2011, in fresh poultry meat the food safety criteria prescribes 'not detected in 25 g' for the target serovars for poultry populations (*Salmonella* Enteritidis and *Salmonella* Typhimurium, including monophasic *Salmonella* Typhimurium strains). For this it is important to know that a serovar found with antigenic formula 1,4,[5],12:i:- is the monophasic variant of *S.* Typhimurium (1,4,[5],12:i:1,2) and not the monophasic variant of another non-target serovar, like *S.* Lagos (1,4,[5],12:i:1,5), *S.* Agama (4,12:i:1,6), *S.* Farsta (4,12:i:e,n,x), *S.* Tsevie (1,4,12:i:e,n,z_{15}), *S.* Gloucester (1,4,12,27:i:l,w), or *S.* Tumodi (1,4,12:i:z_6).

A final distinction between these target and non-target serovars can only be made with a molecular technique. In 2014, it was therefore decided at ISO and CEN level to develop a standard method for the identification of monophasic *S.* Typhimurium based on PCR technique(s). This method should become available in a new part (part 4) of the EN ISO 6579 series (ISO/TS 6579-4).

First working drafts of ISO/TS 6579-4 were developed in a CEN working group by Burkhard Malorny (NRL-*Salmonella* Germany) and include 3 PCR protocols:

- a probe-based multiplex real-time PCR assay (primers and probes published by Maurischat et al. 2015);
- agarose gel-based multiplex PCR assay (primers published by Tennant et al. 2010, and EFSA, 2010);
- agarose gel-based single target PCR assay (primers published by Maurischat et al. 2015).

All three PCR protocols target a genetic sequence to make a distinction between *S.* Typhimurium and monophasic *S.* Typhimurium with other *Salmonella* serovars. The agarose gel-based multiplex PCR assay targets one genetic sequence which is present in isolates expressing the second H phase antigen. The multiplex real-time PCR assay and the agarose gel-based single target PCR assay target two genetic sequences present in isolates expressing the second H phase antigen. An Internal Amplification Control is also used to distinguish real negative results from those due to inhibitory effects during the amplification process.

To check the performance of the three PCR protocols, 172 strains (target and non-target strains) were analysed by the NRL-*Salmonella* in Germany

as well as by the EURL-*Salmonella*. After re-analysing 12 strains which gave different results for one or more PCR protocols between the two laboratories, only four strains still gave different results. This difference was only seen for the gel-based single target PCR and was caused by a weak amplification band of one of the targets, resulting in different interpretation of the results. A lower final concentration of this target was tested (0,2 pmol/µl instead of 0,4 pmol/µl), but still a weak amplification band was formed and the interpretation of the results were similar.

Two adjustments were tested in the protocol of the gel-based multiplex PCR. The amplification time was prolonged to 1,5 min (instead of 1 min), which gave a stronger 1389 bp band formation. The final primer concentration of target *fliA-fliB* and target *fliB,* was changed to 0,4 pmol/µl and 0,2 pmol/µl respectively. This resulted in a lower amount of primer-dimers without influencing the amplification fragments.

Additionally, 22 strains were re-analysed with slide agglutination. These results revealed that 15 of these strains were monophasic *S.* Typhimurium, after originally been serotyped as (biphasic) *S.* Typhimurium. The results of the slide agglutination was in agreement with the results of the multiplex real-time PCR and with the gel-based single target PCR.

The three PCR protocols showed comparable results for the following number of strains (in total 172 strains were tested): 38 monophasic *Salmonella* Typhimurium strains, 39 (biphasic) *Salmonella* Typhimurium strains, 46 strains of other *Salmonella* serovars (not *S* Typhimurium, not monophasic *S.* Typhimurium) and 27 strains of other *Enterobacteriaceae* (not *Salmonella*). Twenty-two strains showed different results between the three PCR protocols in identifying the strains as monophasic *S.* Typhimurium or as biphasic *S.* Typhimurium. The majority of these strains were tested as monophasic *S.* Typhimurium with slide agglutination, with the multiplex real-time PCR, and with the gel-based single target PCR, and as biphasic *S.* Typhimurium with the gel-based multiplex PCR. This can be explained by the fact that the gel-based multiplex PCR does not reflect all regions in the *flj* gene cluster which are associated with second H phase flagellar antigen expression, while the multiplex real-time PCR as well as the gel-based single target PCR do target these regions. For that reason, the gel-based multiplex PCR may be less specific for some strains than the other two PCR protocols.

3.2 Comparison of *Salmonella* Typhimurium and monophasic variants from farms in East Anglia

Katharine Newton, NRL-Salmonella, Weybridge, United Kingdom

Salmonella Typhimurium (STm) and more recently monophasic variants (mSTm) are now occasionally reported in multiple animal species in the UK, being primarily associated with pigs. Isolations of both, STm and mSTm from UK poultry farms are subject to strict controls as part of statutory and industry control programs. Many of Britain’s pig and poultry farms are based in East Anglia. Using high resolution WGS methods, we investigated 35 STm and 78 mSTm isolates from 83 different farms in East Anglia to assess the potential exchange of

STm and mSTm strains between livestock sectors. The majority of isolates were from pigs, turkeys and chickens. Additional isolates from other poultry, cattle, horses and animal feed were also included.

Phylogenetic analyses performed by means of core genome SNP analyses of mSTm isolates showed a distinct clustering of genetically nearly identical isolates from different livestock production sectors. The third level Bayesian classification subdivided the mSTm isolates into a further two sub-clades, G1 and G2. Isolates in sub-clade G1 were more diverse and included 17 of the 19 older mSTm isolates, from other UK regions. Sub-clade G2 consisted mostly of isolates associated with farms in East Anglia. Strikingly, in this cluster 14 isolates (or 25%) differed by only 5 SNPs. A 5 SNP difference is considered to represent the same clone in a *Salmonella* outbreak scenario. The clustered isolates were from pigs, chickens, turkeys and a horse, from 13 different farms in East Anglia. Furthermore, 26 isolates (or 46%) of which 11 isolates were from turkeys, 8 from chickens, 5 from pigs, and single isolates from a horse and animal feed were in the 10 SNP group (i.e. 10 SNPs apart). Overall, 71% of the mSTm isolates were within the 10 SNP cluster, suggesting an evolutionary relatedness of isolates from different farms in East Anglia distinct to mSTm isolates from previous years and other UK regions.

To confirm these observations, we applied a Machine Learning model trained to recognize and associate particular gene features (187 cgMLST loci) with specific primary animal source classes. Based on the training and the test set assignments, the model could accurately distinguish between the poultry and pig primary source classes. When applied to the validation set, the model assigned the majority of the chicken and turkey isolates to the pig primary source class. Of the 59 pig isolates, 50 were assigned to pigs and 9 to other primary sources.

Bayesian evolutionary analysis was conducted to elucidate the phylogeny amongst the 78 mSTm isolates in G2 clade associated with the most parsimonious (simplest with greatest explanatory power) phylogeny generated by BEAST (Bayesian Evolutionary Analysis Sampling Trees), and to visualise the evolutionary history using Spread3. The analysis suggests that infection was largely transferred from pigs to poultry, supporting the Machine Learning findings. In conclusion, these results indicate that in the East Anglia region, the direction of transmission of *Salmonella* was from pig farms to poultry farms, and there was little evidence of *Salmonella* transmission the other way.

Discussion

Q: Do you know if DT193 is Sequence Type 34, the main Sequence Type for monophasic *S.* Typhimurium?
A: Yes.
Q: Do you have any idea on how transmission from pig farms to poultry takes place? What is the supposed vector?
A: In 2018 it was a dry and hot summer and there was a lot of dust transmission from the outdoor pig farms to the poultry farms. We took samples outside the poultry premises and found pig associated *Salmonella* serovars like *S.* Panama. Flies could also be involved, but dust transmission via wind is probably the main vector.

3.3 Verification of methods following EN ISO 16140-3; Theory and practice

Paul in 't Veld, NVWA, Utrecht, the Netherlands
*Wilma Jacobs-Reitsma, EURL-*Salmonella*, Bilthoven, the Netherlands*

In ISO/TC34/SC9 (International Organization for Standardization, Technical Committee 34 on Food Products, Sub-committee 9 – Microbiology), working group 3 (WG3) drafted several parts of the EN ISO 16140 series for the validation of methods for microbiology of the food chain. In 2020, the final draft version (FDIS) of ISO 16140-3 was published (*note: the final version of EN ISO 16140-3 was published in January 2021)*. This document describes a protocol for the verification of reference methods and validated alternative methods. The distinction between validation and verification is defined as follows:

- Validation: Establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled.
- Verification: Demonstration that a validated method performs, in the user's hands, according to the method's specifications determined in the validation study and is fit for its intended purpose.

Currently, 6 parts of the EN ISO 16140 series are available and a scheme has been developed to help the user with the choice of the correct part for the validation/verification procedure. To demonstrate that a laboratory can satisfactorily perform a validated method, verification is needed. The procedure for this verification is described in EN ISO 16140-3.

The procedure for verification of qualitative (detection) methods and quantitative (enumeration) methods described in EN ISO 16140-3 is undertaken in two parts:

- implementation verification, and
- (food) item verification.

The verification focuses on (food) items within the scope of validation and within the scope of laboratory application.
For the implementation verification, the user laboratory shall perform the following steps:

1. review validation data for the method;
2. select one (food) item tested during the validation study that is within the scope of the laboratory application of the user laboratory;
3. use this one (food) item, and the sample size used in the validation study, to perform the implementation.

For the (food) item verification, the user laboratory shall perform the following steps:

- select one challenging (food) item from each (food) category listed within the scope of validation, that is also a (food) category tested within the scope of the laboratory application of the user laboratory, and
- use this one (food) item to perform the (food) item verification.

If the scope of laboratory application is for a 'broad range of foods', then five or more food categories have to be tested in the verification study. Additionally, three more categories have been defined: animal feed (including pet food), environmental samples of food or feed production, and samples from the primary production stage (PPS). If these latter three categories are also part of the laboratory application, then these shall be tested in addition to the five food categories. All categories are defined in annex A of EN ISO 16140-3.

The performance characteristics to be determined are the following:

- For qualitative methods, the estimated LOD_{50} ($eLOD_{50}$: level of detection at 50% probability of detection) shall be determined for the implementation verification as well as for the (food) item verification.
- For quantitative methods, the intra-laboratory reproducibility (S_{IR}) has to be determined for implementation verification and the estimated bias (eBias) for the (food) item verification.

The protocols for determining these performance characteristics are described in EN ISO 16140-3.

The presentation given by Paul in 't Veld on the theory of verification of microbiological methods according to draft EN ISO 16140-3 was followed by a presentation by Wilma Jacobs on how this theory was used in practice at the EURL-*Salmonella* laboratory, located at RIVM in Bilthoven, the Netherlands.

As *Salmonella* detection according to EN ISO 6579-1:2017 is a qualitative method, the $eLOD_{50}$ had to be determined for the implementation verification and for the (food) item verification.
EN ISO 16140-3 describes three protocols for determining the $eLOD_{50}$ and the user can choose one of these protocols. For research reasons only, the EURL-*Salmonella* tested all three protocols.

For the implementation verification, chicken fillet parts were tested, a food item belonging to the category 'raw poultry & ready-to-cook poultry products'. For the (food) item verification, a total of 8 items from 8 different categories were tested: 5 food categories and 3 additional categories (animal feed, environmental samples, and samples from the primary production stage).

For data analysis, ISO/TC34/SC9-WG3 developed an Excel tool which was also used to analyse the data of this verification study. This Excel tool indicates if results are accepted or not, based on the criteria defined in EN ISO 16140-3. If a result is not accepted, it may be necessary to repeat the analysis for this (food) item.

Note: In March 2021 supporting materials to facilitate the implementation of EN ISO 16140-3 became available on the ISO/TC34/SC9 website: https://committee.iso.org/sites/tc34sc9/home/essential-information/content-left-area/validation-of-methods/method-validation-and-method-ver.html

Discussion

Q: After EN ISO 16140-3 has been published, do we need to perform re-verification for existing methods (accredited) in the laboratory, or do we apply this norm only for newly applied methods in the laboratory?
A: A transition document will be made available, describing when to apply EN ISO 16140-3. For current methods already validated/verified by the laboratory, no re-verification is needed. Only in cases where a laboratory want to add new methods to the scope of accreditation, EN ISO 16140-3 shall be followed.
Q: Would we have to do an implementation verification if we chose one food category that wasn't included in the validation study of the reference method?
A: When you verify a method for only one (food) category not used in the validation study, you still need to carry out the implementation verification and select one of the items used in the validation study. For all cases, an implementation verification and a food item verification has to be performed to demonstrate the application of the method.
Q: Which LOD_{50} calculation Excel sheet do you use?
A: You do not need to perform a calculation as the information is given in tables.
Q: Is the ISO spreadsheet for entering results available to everyone?
A: Not yet, as it is under preparation. However, the link will be included in the final EN ISO 16140-3 publication and the link to the tool will also become available on the ISO/TC34/SC9 website.
Q: What if the food category isn't included in the initial validation of the reference method? Would it be a validation then?
A: If the method is validated for a broad range of foods, then it would be an item verification. However, if the method is validated for a limited number of (food) categories and this food category was not included, then it will be a validation.
Q: If the laboratory uses a different portion size to the one used in the validation study (e.g. 25g instead of 10g), does the laboratory then have to do a validation instead?
A: Yes. A protocol will be drafted for validation of larger test portions.
Q: EN ISO 16140-4 directs to EN ISO 16140-2, which is a lot of work for one laboratory?!
A: This reference is made only to the method comparison part of EN ISO 16140-2. EN ISO 16140-4 describes a (in-house) validation and not a verification, which is more work. After validation in accordance with EN ISO 16140-2, no verification is needed anymore.
Q: What do you actually mean by 'challenging' food categories? I think this could be rather subjective?
A: Indeed, a 'challenging' food category is subjective. The intention is that the laboratory is confident that it can apply the method well, but it is indeed difficult to say what is challenging.
Q: When is the protocol for validating a larger test portion size due? Will this be part of the EN ISO 16140 series?

A: This protocol is still under development and it is not yet sure if it will become part of the EN ISO 16140 series. The annex of EN ISO 6887-1:2017 already describes a protocol for how to test the pooling of samples. For the moment, this protocol could be used for testing larger test portions as well.

3.4 Work programme EURL-*Salmonella* second half 2020, first half 2021, discussion on general items and closure

Kirsten Mooijman, head EURL-Salmonella, Bilthoven, the Netherlands

Kirsten Mooijman summarised the information on the work programme of the EURL-*Salmonella* for the rest of 2019 and for early 2020.

In December 2018, the EURL-*Salmonella* submitted a two-year work programme (2019–2020) to EC DG SANTE. The template for the work programme follows Regulation EU No 625/2017 (EC, 2017), Article 92 (2). Approval by DG SANTE of the work programme and the budget for 2019–2020 was received in April 2019.

Activity 1 To ensure availability and use of high-quality methods and to ensure high-quality performance by NRLs
Sub-activity 1.1 Analytical methods
Objectives:

- to standardise methods (ISO and CEN);
- to keep track of developments in (alternative) methods;
- to provide NRLs with information on developments of relevant (standardised/new) analytical methods.

This activity includes the activities for ISO and CEN:

- ISO-Ad hoc group on drafting Amd.1 of EN ISO 6579-1 (project leader) has been finalised. In March 2020, EN ISO 6579-1:2017/Amd.1:2020 was published.
- ISO-WG10 (convenor) – drafting CEN ISO/TS 6579-4 'Microbiology of the food chain - Horizontal method for the detection, enumeration and serotyping of *Salmonella* - Part 4: Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR)'. From 18 May until 16 August 2020, the voting for the New Work Item Proposal (NWIP) for draft CEN ISO/TS 6579-4 took place. The outcome was 100% approval in ISO and CEN with few comments. The next steps are to prepare one or more working drafts (WDs) in ISO format and discuss this with the members of ISO-WG10 (meetings planned in November 2020 and March 2021). This document will become a Technical Specification (TS) which has a limited number of voting rounds. After agreement in ISO-WG10, a draft Committee Draft (CD) version will be sent to the members of ISO/TC34/SC9 and CEN/TC463 for voting. After incorporating the comments from this voting round, the final draft CD will be prepared and used for an interlaboratory study to set the performance characteristics (probably to be organised in 2022).
- ISO-WG3 Method validation (project leader and member). In December 2019, EN ISO 16140-6 was published ('Protocol for the validation of alternative (proprietary) methods for microbiological

confirmation and typing procedures'). In July 2020, parts 4 ('Protocol for single-laboratory (in-house) method validation') and 5 ('Protocol for factorial interlaboratory validation of non-proprietary methods') of EN ISO 16140 were published. The voting for the Final Draft International Standard (FDIS) of part 3 ('Protocol for the verification of reference and validated alternative methods implemented in a single laboratory') took place from 2 October until 27 November 2020 (*note: the final version of this document was published in January 2021)*. In 2020, the revision of EN ISO 17468 ('Microbiology of the food chain - Technical requirements and guidance on establishment or revision of a standardized reference method') started. This activity will be continued in the coming years.

- ISO-AHG1 (project leader) on harmonisation of ISO/CEN standards for microbiology of the food chain: updating guidance document for publication of edition 3 in 2021.
- ISO-AHG4 (member) on validation status of ISO/CEN standards: making an inventory on whether EN ISO documents of the Food chain contain (complete) performance characteristics or not.
- ISO-WG25 Whole genome sequencing (member). Development of EN ISO 23418 'Microbiology of the Food Chain - Whole genome sequencing for typing and genomic characterization of foodborne bacteria - General requirements and guidance'. The voting for the Draft International Standard (DIS) took place from 18 September until 11 December 2020. The comments will be discussed at a meeting of ISO-WG25 in spring 2021.

Sub-activity 1.2 joint EURLs working group on NGS
Objectives:

- to promote the use of NGS across the EURL networks;
- to build capacity for producing and using NGS data within the EU;
- to ensure liaison between the work of the EURLs and the work of EFSA and ECDC on NGS.

The working group includes 8 biological EURLs, and 8 activities have been defined in relation to NGS. For each activity, guidance documents will be prepared and published on the EURLs' websites. On 25 September 2021, an online conference was organised with support of the Med-Vet-Net association 'Modern technologies to enable response to crises: Next Generation Sequencing to tackle food-borne diseases in the EU'. The EURLs' working group will also organise training courses. However, due to the SARS-CoV-2-virus pandemic, the training course on NGS of 2020 is postponed to 2021.

Sub-activity 1.3 Proficiency Tests
Objective:
Organisation of Proficiency Tests (PTs) to gain information on the performance of the NRLs-*Salmonella* for detection and typing of *Salmonella.*

In the coming year, three PTs are foreseen:

1. Detection of *Salmonella* in samples from the primary production stage. This study will be held in September/October 2020 and concerns a combined PT for Primary Production Stage (PPS) and Food, on detection of *Salmonella* in hygiene swabs. NRLs-*Salmonella* analysing PPS samples as well as Food samples can participate (obligatory for NRLs-PPS).
2. Detection of *Salmonella* in food samples. This study is foreseen for March 2021. The matrix for this study has not yet been decided.
3. Typing of *Salmonella* (serotyping, molecular typing). This study is foreseen for November 2020 and will include serotyping of *Salmonella* (obligatory) and a second pilot for cluster analysis for which a molecular method free of choice can be used (PFGE and/or MLVA and/or WGS).

Activity 2 To provide scientific and technical assistance to NRLs

Sub-activity 2.1 Workshop

Objective:

To exchange information on the activities of the NRLs-*Salmonella* and the EURL-*Salmonella* and on (new) developments in the relevant work field.

Whether the 2021 workshop will again be organised as an online meeting or as a physical meeting depends on the situation with the SARS-CoV-2-virus pandemic. The workshop will probably be organised by the end of May 2021.

Sub-activity 2.2 Training courses

Objective:

To train NRLs-*Salmonella* in a specific work field.

The physical training courses will depend on the situation with the SARS-CoV-2-virus pandemic and may concern:

1. training on request of an NRL (requests for 2020 are postponed to 2021);
2. training following advice from the EURL (e.g. in case of repeated poor performance in PTs);
3. joint EURLs training on WGS (basics), organised in cooperation with other EURLs (postponed from 2020 to 2021).

Sub-activity 2.3 Scientific advice and support of NRLs

Objectives:

- to provide scientific and technical assistance to the NRLs-*Salmonella* for the relevant work field;
- to perform confirmatory testing (samples/isolates) for NRLs when needed;
- to perform WGS analysis of isolates of NRLs-*Salmonella* for outbreak investigations;
- to maintain the EURL-*Salmonella* website and keep the information on the website up to date;
- to inform NRLs on the activities of the EURL and other parties in the relevant work field, as well as on developments in this field;
- to publish four newsletters per year, through the website.

Activity 3 To provide scientific and technical assistance to the European Commission and other organisations
Sub-activity 3.1 Scientific advice and support of EC and other organisations
Objectives:

- to provide scientific and technical assistance to EC DG SANTE for the relevant work field;
- to provide assistance to DG SANTE, EFSA, and (NRLs of) Member States in the event of (international) *Salmonella* outbreaks;
- to collaborate with EFSA and ECDC for the relevant work field;
- to cooperate with other biological EURLs.

Description:

- ad hoc scientific and technical assistance of DG SANTE;
- participation in working groups/scientific committees of DG SANTE and EFSA, such as the EFSA–ECDC Steering Committee of the molecular database;
- assistance of DG SANTE, EFSA, NRLs, and ECDC in the event of outbreaks, e.g. consultation of NRL network for specific information, (sub)typing of suspect isolates (MLVA, NGS), and analysis of data.

Activity 4 Reagents and reference collections
Sub-activity 4.1 Reference strains and reference materials
Objective:
To supply information on available culture collections and suppliers of microbiological reference materials.

Description:

- providing a link to the White-Kauffmann-Le Minor scheme and keeping contacts with WHO reference centre;
- reference to culture collections and reference materials at the website;
- maintenance of in-house culture collection;
- provision of sets of reference strains (*S.* Enteritidis and *S*. Typhimurium) for MLVA typing;
- sub-activity 4.1 is merged with 2.3 (supporting NRLs; keeping information on website up to date).

4 Evaluation of the workshop

4.1 Introduction

At the end of the workshop, a link to an evaluation form was sent to the participants asking them for their opinion by answering 11 questions (see Annex 3). For several questions, participants were asked to give a score ranging from 1 to 5. The scores represent: very poor (1), poor (2), fair (3), good (4) and very good (5). In addition, it was possible to add comments. Two questions were 'open' questions, in which the participants were asked to give their opinion.

The evaluation form was sent to all participants, but the staff members of the EURL-*Salmonella* were excluded from the evaluation, making a total of 68. In total, 36 Participants completed the evaluation form, a response rate of 53%. This is a lower response rate compared to earlier workshops where the rate was generally >80%. An explanation for the lower response rate might be that for the current workshop, the participants had to login to complete the evaluation form, while at earlier workshops the evaluation form was handed over (on paper) at the end of the workshop. Still, this evaluation represents the opinion of more than half of the number of participants and gives valuable information.

In section 4.2, the scores given to each question are presented and a summary of the remarks is given.

4.2 Evaluation form

1. What is your opinion on the information given in advance of the workshop?
Figure 1 shows that the majority of respondents considered the information given in advance of the workshop as very good (score 5). One participant remarked that 'the amount of information was perhaps excessive'.

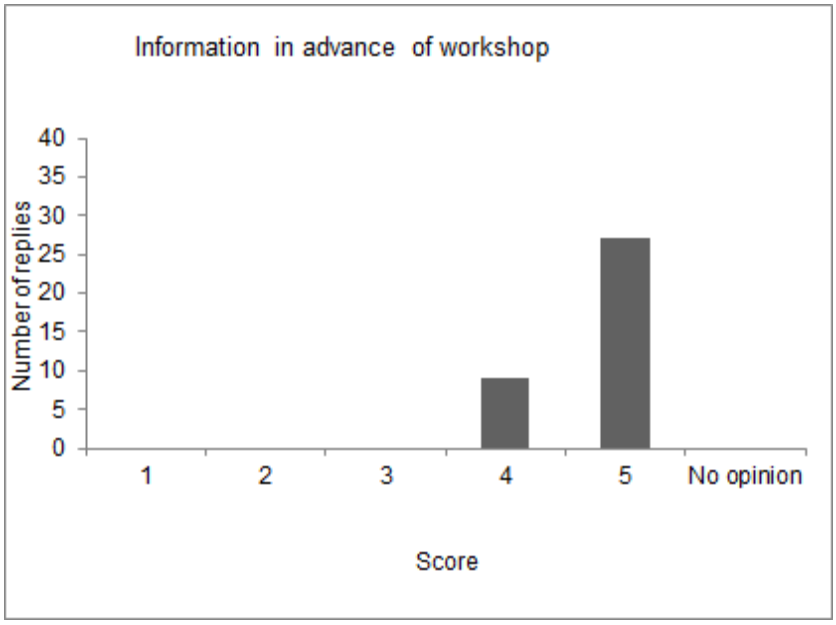

Figure 1 Scores given to question 1 'Opinion on information given in advance of the workshop'

2. What is your opinion on the easiness to login into the meeting?
The majority of the participants found it easy to login into the online meeting (see Figure 2). A remark related to this question was: 'GoToMeeting is the best platform for holding virtual meetings and worked very well'.

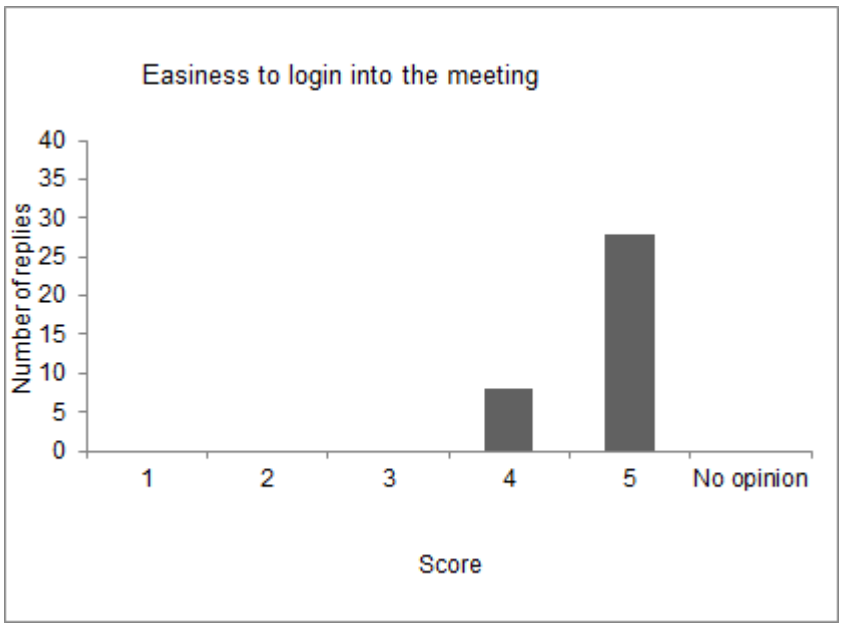

Figure 2 Scores given to question 2 'Opinion on the easiness to login into the meeting'

3. Did you face any technical problems during the meeting?
Only 3 of the 36 respondents faced technical problems during the meeting (see Figure 3). These 3 technical problems concerned: 'loss of internet', 'problems with sound and image (shortly)', 'little problems with audio'.

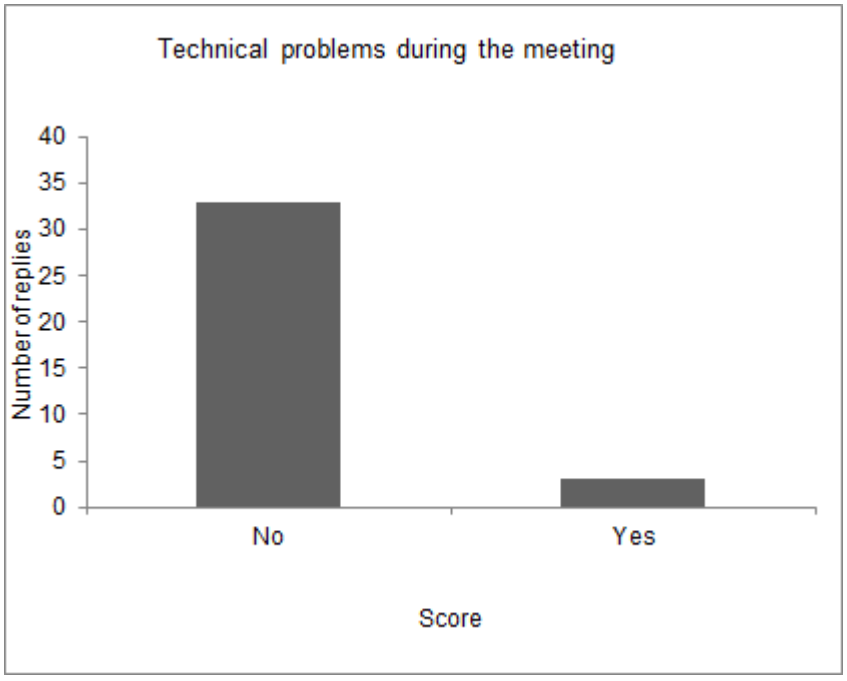

Figure 3 Replies given to question 3 'Did you face any technical problems during the meeting?'

4. What is your opinion on the length of the meeting and the number of breaks?
33 of the 36 respondents considered the length of the meeting to be fine (Figure 4a) and 30 of the 36 respondents considered the number of breaks to be fine (Figure 4b). One remark was made, being: 'well-planned meeting.'

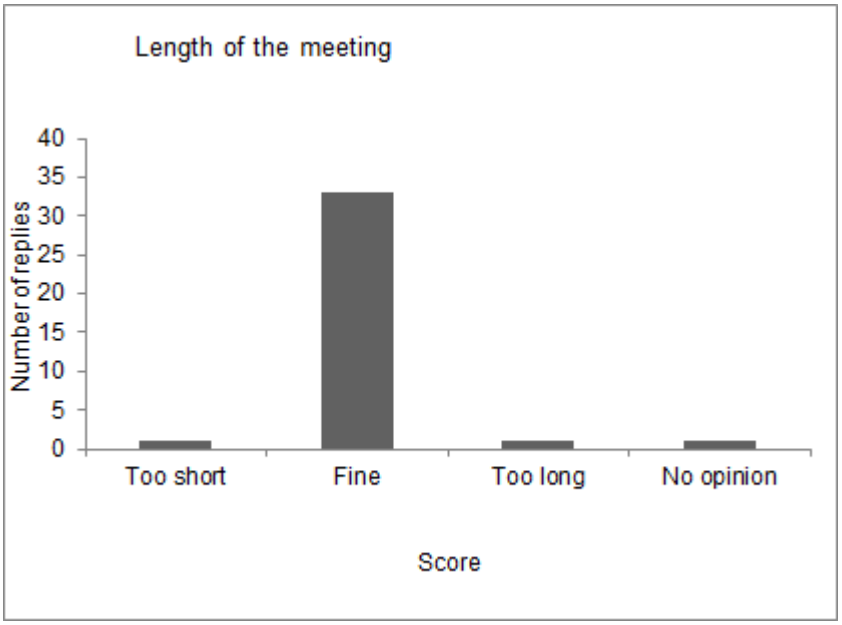

Figure 4a Replies given to question 4 'What is your opinion on the length of the meeting?'

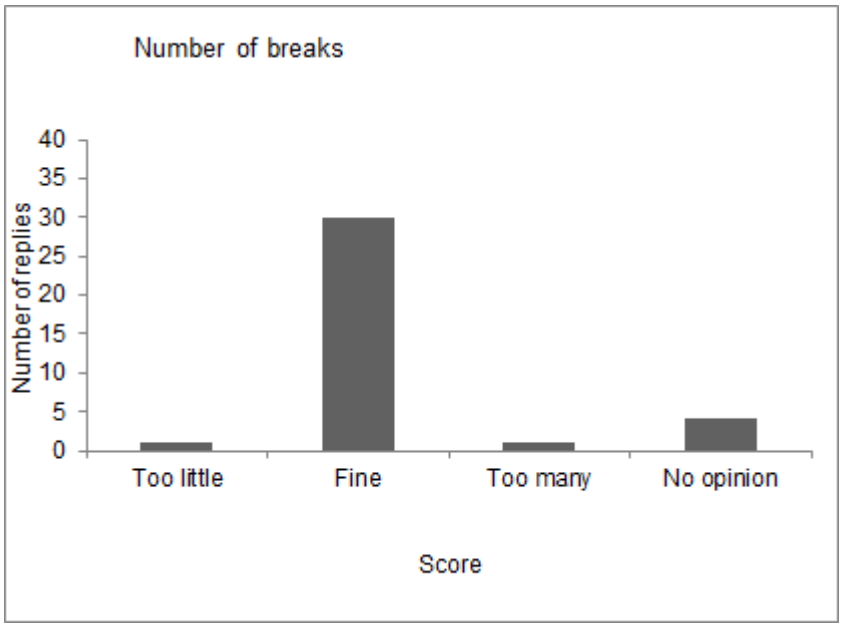

Figure 4b Replies given to question 4 'What is your opinion on the number of breaks?'

5. Were you satisfied with the options for raising questions during the meeting (chat function; discussion time at the end of a presentation and at the end of a session)?
32 of the 36 respondents were satisfied with the options for raising questions. The remaining 4 respondents had no opinion (Figure 5). The following remarks were made:
'Discussion was fairly poor compared with the workshops with physical attendance. It is only normal, as you need time to read the question, answer back, etc.'
'Maybe one could give a sign for a question in the chat. Then the Chair can give the floor to the person with a question, so that each person can raise the question via the microphone. For some specific, longer questions, the chat is not comfortable, also because some participants may have problems with correct spelling.'

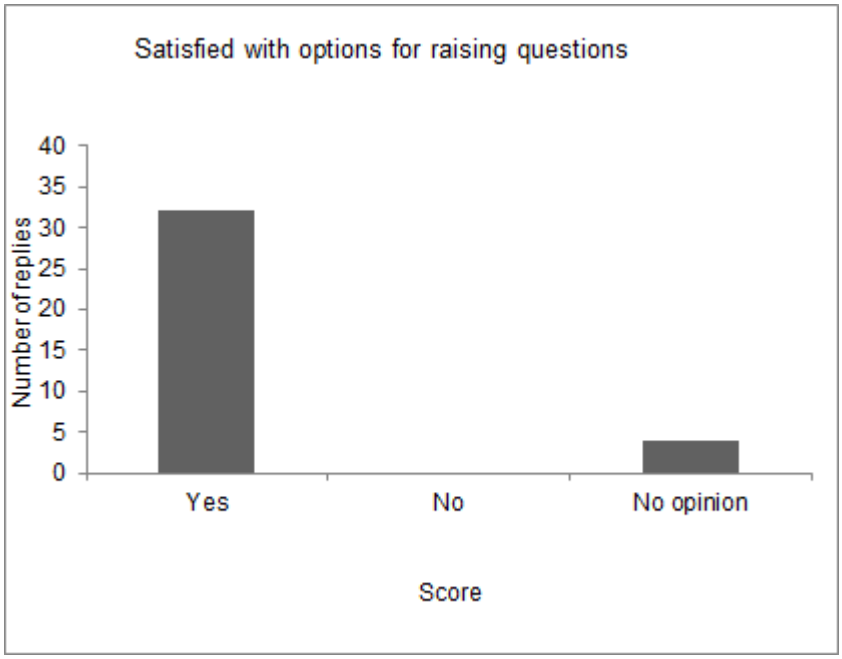

Figure 5 Scores given to question 5 'Were you satisfied with the options for raising questions during the meeting?'

6. What is your opinion on the scientific programme of the workshop?
The majority of respondents were satisfied with the workshop's scientific programme; the majority of the scores were good (4) to very good (5), see Figure 6. It was remarked that 'all talks were of relevance'.

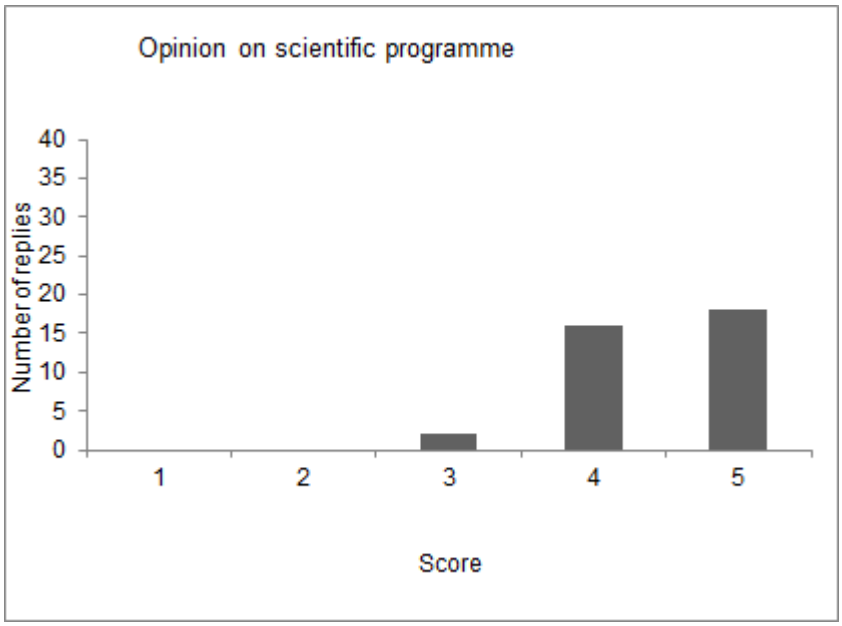

Figure 6 Scores given to question 6 'What is your opinion on the scientific programme of the workshop?'

7. Are there specific presentations you want to comment on, or did you miss information on certain subjects?
This was an 'open' question and a few responded 'no', or 'not applicable'. Only one remark was given: 'I did miss some information (because we had some technical problems with our server), but I hope than I will find all necessary information and presentations on the EURL home page.'

8. What is your general opinion of the workshop?
All but 1 respondents indicated that the workshop as a whole had been good (4) or very good (5), see Figure 7.

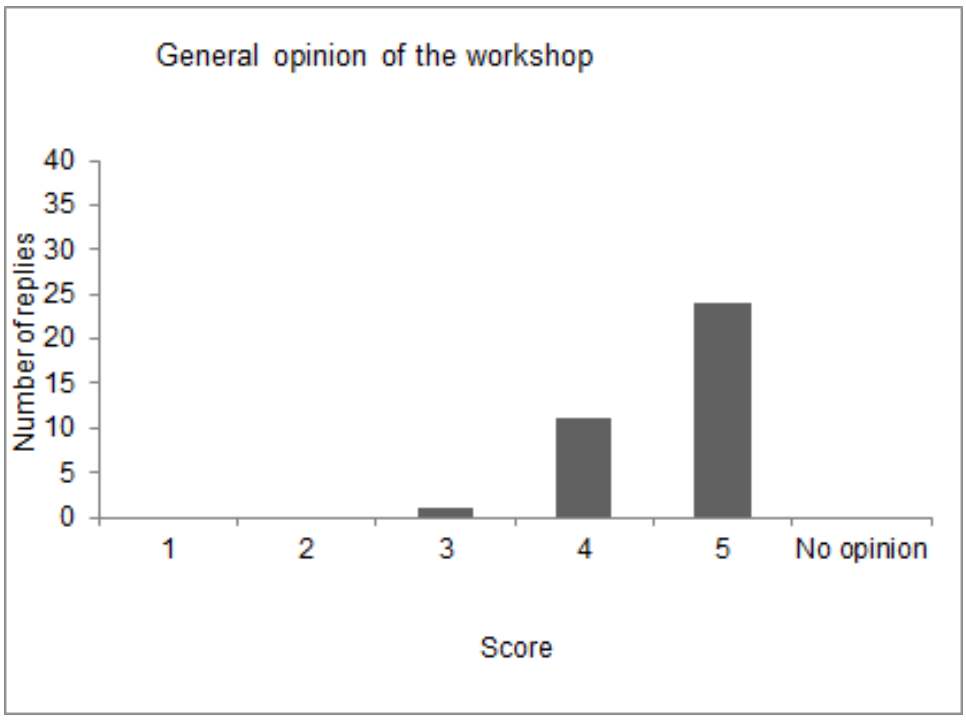

Figure 7 Scores given to question 8 'What is your general opinion of the workshop?'

9. Do you consider an online workshop as a good alternative for a physical meeting?
Of the 36 respondents, 19 considered an online workshop as a good alternative for a physical meeting, 9 disagreed, and 8 reported having no opinion (see Figure 8).

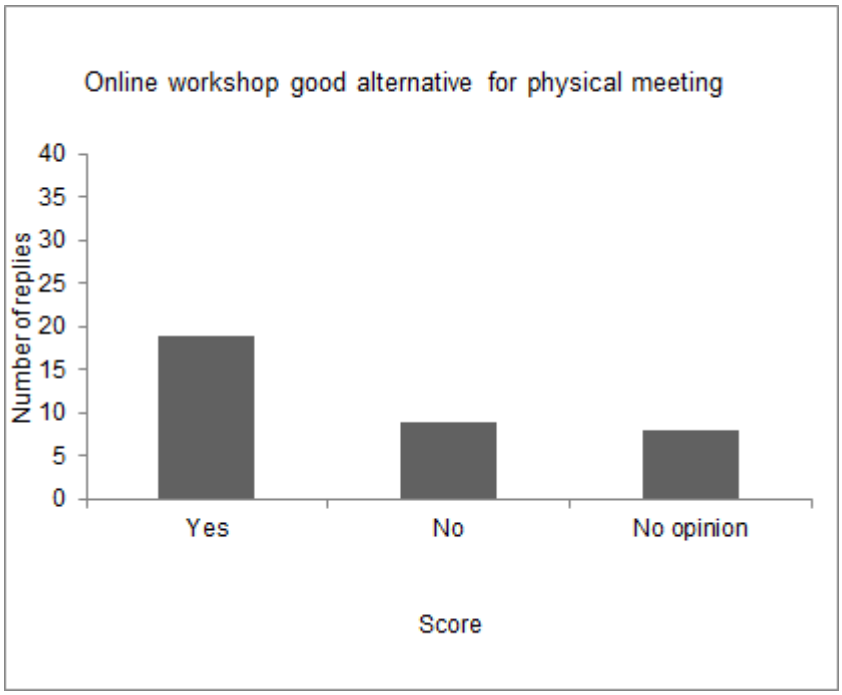

Figure 8 Replies given to question 9 'Do you consider an online workshop as a good alternative for a physical meeting?'

Several remarks were made in answer to question 9:
'It is an opportunity for people of multi-disciplines to participate, which is a definite advantage over the restrictions posed with face-to-face meetings where numbers have to be limited. And the technology could handle the extra participants without problems.'
'The physical meeting provides the opportunity for networking while the online workshop just provides scientific knowledge.'
'It was very good, but a physical meeting is always better.'
'Maybe every two years you could consider to have an online workshop. Physical meetings cannot be replaced. Face to face communication is still important for establishing strong cooperation between participants and EURL.'
'I consider an online workshop as a good alternative for a physical meeting, but a physical meeting comes definitely first!'
'In case there are factors that prevent us from having physical meetings, yes.'

10. Should we organise more online meetings instead of physical meetings, even in non-pandemic times?
The opinions of the respondents to this question were mixed. Approximately half of the respondents were positive about the idea to organise more online meetings, while the other half was negative (see Figure 9). The following remarks were made:

'I think that in the future, every other meeting could be an online version. This saves both time and of course money and CO_2.'
'Only if these meetings deal with specific subjects and do not represent an alternative for the annual workshop.'
'There is no substitute for physical meetings with colleagues with the possibility of exchanging experiences and opinions face to face.'
'It could be a good idea to alternate online with physical meetings.'
'yes, but in addition to at least one physical workshop a year.'

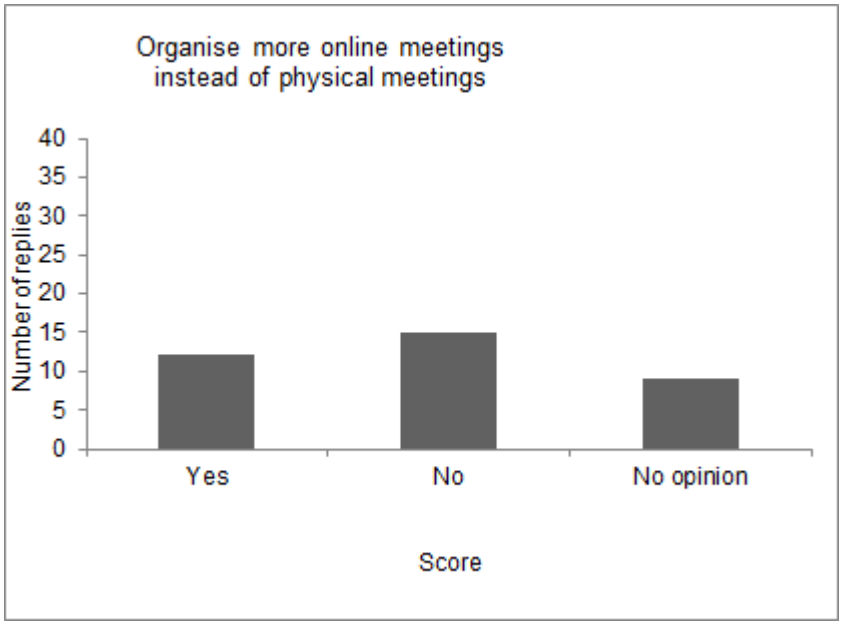

Figure 9 Replies given to question 10 'Should we organise more online meetings instead of physical meetings, even in non-pandemic times?'

11. Do you have any remarks or suggestions which we can use for future workshops?
This was another 'open' question and the following responses were received:
'Given that the technology coped very well with this large number of participants, I would like to see the option of holding a virtual workshop on an alternate basis in the future (i.e. one year face-to-face, the next year virtual).'
'Virtual meetings give experts from multi-disciplines (i.e. food, animal, characterisation, detection) the chance to participate, while for 'normal' physical meetings choices for participation need to be made due to the limitations of space and budgets.'
'The Quiz was a great idea.'
'Online workshops cannot be a substitute for physical workshops. There is very little chance (slim to none) for networking. And being away from your work place gives the possibility to concentrate in full to the workshop (no work distractions form the colleagues in the office).'
'Consider to organise the workshop as an online meeting every two/three years. Also, it would be very helpful if participants who can't attend (physically) would have the chance to participate on line. This is a great tool for the future.'

'Thanks for the great workshop. It went really professional, as if you would have years of experience with web conferences.'
'The program was great. I will be happy though to hear the single NRL presentations again in one of the next meetings.'
'It is important for me to see the speakers when they are giving presentations. helps you get the message better.'
'Colleagues, especially English colleagues, should speak more slowly, in order to better follow the message. Otherwise, everything worked very well.'

4.3 Discussion and conclusions of the evaluation

Due to the worldwide SARS-CoV-2-virus pandemic, it was not possible to organise a physical workshop in 2020. However, the online workshop was considered a good alternative, although many participants indicated their preference for a physical meeting. Still, it was also indicated that it may be worthwhile considering the organisation of an online workshop every 2 or 3 years, so that a larger audience can access the workshop. In general, the participants were satisfied with the organisation, technical aspects, and with the scientific programme of this first online EURL-*Salmonella* workshop.

Acknowledgements

The author would like to thank several persons for their valuable help with the organisation of the workshop.
Noël Peters for taking care of the registrations and all other administrative aspects concerning the workshop.
The EURL-*Salmonella* staff - Robin Diddens, Wilma Jacobs-Reitsma, Angela van Hoek, and Irene Pol-Hofstad – are thanked for their valuable help with the organisation of the workshop.
Robin Diddens is specially thanked for his technical support during the meeting, making sure that all presentations were fluidly shared with the audience.
Wilma Jacobs-Reistma is specially thanked for organising the great quiz about *Salmonella* serovars, and for preparing the break presentations and the 'online group picture'.

List of abbreviations

A	Answer
AHG	Ad hoc group
AMR	Antimicrobial resistance
BLAST	basic local alignment search tool
BPW	Buffered Peptone Water
CD	Committee Draft
CEN	European Committee for Standardization
cfu	colony forming units
cgMLST	core genome Multi-Locus Sequence Typing
CI	Confidence Interval
DG SANTE	Directorate-General for Health and Food Safety
DIS	Draft International Standard
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EFTA	European Free Trade Association
$eLOD_{50}$	estimated level of detection at 50% probability of detection
ESBL	Extended Spectrum Beta-Lactamase
EU	European Union
EURL	European Union Reference Laboratory
FDIS	Final Draft International Standard
ISO	International Organization for Standardization
ISO/TC34/SC9	International Organization for Standardization, Technical Committee 34 on Food Products, Sub-committee 9 – Microbiology
LBM	Live Bivalve Molluscs
MDR	Multi drug resistant
MKTTn	Mueller Kauffmann Tetrathionate broth with novobiocin
MLST	Multi-Locus Sequence Typing
MLVA	Multi-Locus Variable number of tandem repeats Analysis
MPN	Most Probable Number
MS	Member State
mSTm	monophasic *Salmonella* Typhimurium
MSRV	Modified Semi-solid Rappaport Vassiliadis
NGS	Next Generation Sequencing
NRC	National Reference Centre
NRL	National Reference Laboratory
NVWA	Netherlands food and consumer product safety authority
NWIP	New Work Item Proposal
OL	Official Laboratory
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PPS	Primary Production Stage
PT	Proficiency Test
Q	Question
qPCR	quantitative Polymerase Chain Reaction
RASFF	Rapid Alert System for Food and Feed
RIVM	National Institute for Public Health and the Environment
ROA	Rapid Outbreak Assessment

RVS	Rappaport Vassiliadis broth with Soya
SC	Sub Committee
SNP	Single-Nucleotide polymorphism
STEC	Shiga toxin-producing *Escherichia coli*
STm	*Salmonella* Typhimurium
TC	Technical Committee
TS	Technical Specification
UK	United Kingdom
WG	Working Group
WGS	Whole Genome Sequencing
WKLM	White Kauffmann Le Minor

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Annex 1 Participants

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European Food Safety Authority (EFSA)	Frank Boelaert Valentina Rizzi Mirko Rossi
EURL-*Salmonella*	Robin Diddens Angela van Hoek Wilma Jacobs-Reitsma Kirsten Mooijman Noël Peters Irene Pol-Hofstad

Guest speakers

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Sweden	Joakim Skarin (EURL-*Campylobacter*, Uppsala)

National Reference Laboratories for *Salmonella*

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AUSTRIA	Christian Kornschober Heimo Lassnig
BELGIUM	Sarah Denayer Maria Cristina Garcia Graells
BOSNIA HERZEGOVINA	Amira Koro Emina Residbegovic
BULGARIA	Gergana Mateva Mihail Milanov
CROATIA	Borka Simpraga
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	Paulina Pasim
PORTUGAL	Hugo Guedes
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	Mirko Prodanov
ROMANIA	Carmen Manea
SERBIA	Jasna Kureljusic
	Nemanja Zdravkovic
SLOVAK REPUBLIC	Lubos Mikula
	Katarina Rostakova
SLOVENIA	Jasna Micunovic
	Tina Pirs
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	Maria Cristina de Frutos
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	Jenny Eriksson
SWITZERLAND	Guido Bloemberg
UNITED KINGDOM	Katharine Newton
	Amisha Vibhakar
UNITED KINGDOM -NORTHERN IRELAND	Angela Lahuerta Marin

Annex 2 Workshop Programme

Programme of the 25th EURL-*Salmonella* workshop
17 and 18 September 2020 - Online

Thursday 17 September 2020

Morning session: Proficiency Tests

09:30 - 10:00	Opening and introduction	Kirsten Mooijman EURL-*Salmonella*
10:00 - 10:30	Results EURL-*Salmonella* Proficiency Test Primary Production 2019 - Detection of *Salmonella* in chicken faeces samples	Irene Pol EURL-*Salmonella*
10:30 - 10:45	*Break*	
10:45 - 11:15	Preliminary results EURL-*Salmonella* Proficiency Test Live Bivalve Molluscs 2020 Detection of *Salmonella* spp. in mussels	Robin Diddens EURL-*Salmonella*
11:15 - 11:45	Results EURL-*Salmonella* Proficiency Test Typing 2019 – serotyping and cluster analysis	Wilma Jacobs EURL-*Salmonella*
11:45 - 12:00	Concluding discussion morning session	
12:00 – 13:30	*Break*	

Afternoon session: NGS

13:30 - 14:00	Cluster analysis of WGS-data	Joakim Skarin EURL-*Campylobacter*
14:00 - 14:30	European Commission mandate on 'One Health' system for the collection and analysis of whole-genome sequencing (WGS) data from food/animal isolates	Mirko Rossi EFSA
14:30 - 14:45	*Break*	
14:45 - 15:15	*Salmonella* Enteritidis outbreak in a hotel school in Belgium	Sarah Denayer Belgium
15:15 - 15:45	Whole genome sequence comparison of MDR *Salmonella* Infantis isolates from broilers and humans in the Netherlands	Angela van Hoek EURL-*Salmonella*
15:45 - 16:00	Concluding discussion afternoon session	

Friday 18 September 2020

Morning session: Analytical methods

09:30 - 10:00	Development and testing of draft ISO/TS 6579-4: Identification of monophasic *Salmonella* Typhimurium	Robin Diddens EURL-*Salmonella*
10:00 - 10:30	Comparison of *Salmonella* Typhimurium and monophasic variants from farms in East Anglia	Katharine Newton United Kingdom
10:30 - 10:45	*Break*	
10:45 - 11:30	Verification of methods following EN ISO 16140-3; Theory and practice	Paul in 't Veld The Netherlands Wilma Jacobs EURL-*Salmonella*
11:30 - 12:00	Work programme EURL-*Salmonella* second half 2020, first half 2021 Concluding remarks workshop and closure	Kirsten Mooijman EURL-*Salmonella*

---------- End workshop----------

Annex 3 Workshop evaluation form

Evaluation of the online 25th EURL-*Salmonella* workshop, 17 and 18 September 2020

We would highly appreciate if you could give us your opinion on the 25th EURL-*Salmonella* workshop, organised as online meeting on 17 and 18 September 2020. Thank you very much in advance for completing the questionnaire by 30 September 2020 at the latest.

1. What is your opinion on the information given in advance of the workshop?

1 (Very poor)	2 (poor)	3 (fair)	4 (good)	5 (very good)	No opinion

Remarks:_____

2. What is your opinion on the easiness to login into the meeting?

1 (Very poor)	2 (poor)	3 (fair)	4 (good)	5 (very good)	No opinion

Remarks:_____

3. Did you face any technical problems during the meeting?
   - ☐ No
   - ☐ Yes, I encountered the following problems: _____

Remarks:_____

4. What is your opinion on the length of the meeting and the number of breaks?

Length meeting:
- ☐ Too short
- ☐ Fine
- ☐ Too long

Number of breaks:
- ☐ Too little
- ☐ Fine
- ☐ Too many

Remarks:_____

5. Were you satisfied with the options for raising questions during the meeting (chat function; discussion time at the end of a presentation and at the end of a session)?
   - ☐ Yes
   - ☐ No opinion
   - ☐ No, but I have a suggestion for improvement _____

Remarks:_____

6. What is your opinion on the scientific programme of the workshop?

1 (Very poor)	2 (poor)	3 (fair)	4 (good)	5 (very good)	No opinion

Remarks:_____

7. Are there specific presentations you want to comment on, or did you miss information on certain subjects?

8. What is your general opinion of the workshop?

1 (Very poor)	2 (poor)	3 (fair)	4 (good)	5 (very good)	No opinion

Remarks:_____

9. Do you consider an online workshop as a good alternative for a physical meeting?
   ☐ Yes
   ☐ No opinion
   ☐ No

Remarks:_____

10. Should we organise more online meetings instead of physical meetings, even in non-pandemic times?
    ☐ Yes
    ☐ No opinion
    ☐ No

    Remarks:_____

11. Do you have any remarks or suggestions that we can use for future workshops?

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