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DTU

FWD AMR · RefLabCap

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Report on the third annual in vitro external quality assessment scheme for WGS-based resistome profiling of Salmonella and Campylobacter

EQA3-WGS-AMR

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1. Background and aim

This report presents the third External Quality Assessment for WGS-based resistome profiling in antimicrobial-resistant *Salmonella* and *Campylobacter* (EQA3-WGS-AMR). The EQA is third out of three planned EQAs, organized by Statens Serum Institut (SSI) in collaboration with the Technical University of Denmark (DTU) in the FWD AMR-RefLabCap project.

The aim of this EQA was to compare the participant's ability to identify genes and point mutations (PMs) that confer antimicrobial resistance in *Salmonella* and *Campylobacter* using whole genome sequencing (WGS), based on provided DNA samples.

In order to be able to evaluate the impact of the quality of sequencing performed by each participant on the ability to detect genes and PMs, all participants were asked to use the same tool and database, threshold and reporting approach, as described in the EQA3 protocol (Section 9.1.1), that was shared with all participants.

In addition to that, the participants were recommended to follow the analytical guidelines described in the protocol (https://www.fwdamr-reflabcap.eu/resources/reflabcap-protocols-and-guidelines) that was developed in the FWD AMR-RefLabCap project. Participation in the EQA3-WGS-AMR enabled the participants to identify strengths and weaknesses in their technical and analytical setup and implement improvements, if needed.

DNA from three isolates of *Salmonella* and three isolates of *Campylobacter* was included in this EQA. Forty-one laboratories from the FWD AMR-RefLabCap network were invited to participate. Thirty-eight laboratories accepted the invitation and 30 participants submitted results. The participants represented a total of 25 countries, including nine priority countries.

2. Materials and methods

2.1. Phenotypic testing

The isolates were phenotypically tested for antimicrobial susceptibility by determination of MIC values and subsequent classification as wild type (WT) or non-wild type (NWT) using epidemiological cut-off values (ECOFFs), when available (1). MIC determination was performed following the harmonised EU AST protocol using microbroth dilution method with EUVSEC3 TREK panels from Thermo Scientific, Denmark for *Salmonella* and EUCAMP3 panels for *Campylobacter* (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020D1729&from).

The *Salmonella* panels included the following antimicrobials: amikacin, ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, trimethoprim and trimethoprim-sulfamethoxazole. For *Campylobacter*, the panels included chloramphenicol, ciprofloxacin, ertapenem, erythromycin, gentamicin and tetracycline. The results of phenotypic testing are shown in Table 1 and Table 2. The selection of antimicrobials tested was based on the priority list of antimicrobial agents set in the harmonised EU AST protocol (1), recommended by the European Centre for Disease Prevention and Control (ECDC).

2.2. Sample characterization

The samples used in this EQA3-WGS-AMR were obtained from isolates that represent a wide array of antimicrobial resistance (AMR) markers. The genotypic and phenotypic AMR features of each sample are shown in Table 1 and Table 2.

The WGS-based predicted phenotype can only be directly compared to laboratory-based phenotype if the predicted phenotype, along with the associated genetic determinants for the antimicrobials tested in the laboratory, are available in the database used for phenotype prediction. Additionally, the ECOFFs, set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), have to be available for the antimicrobial in question in order to determine the laboratory-based phenotype.

In most cases in this EQA, it was possible to compare the phenotypic predictions for the tested antimicrobials with the laboratory-established phenotypes for the test isolates. However, some isolates included in this EQA harbour genes or point mutations that confer resistance towards antimicrobials that were not tested phenotypically in the laboratory. Additionally, there are phenotypes for which the genetic determinants have not been elucidated.

For these reasons, it was not possible to determine the correlation between phenotype and predicted phenotype for several antimicrobials, for example azithromycin, cefepime, sulfamethoxazole and tobramycin for *Salmonella* and streptomycin and nalidixic acid for *Campylobacter*. The known phenotype-genotype correlations are described below in each Table.

Table 1. Genotypic and phenotypic characteristics of the Salmonella samples selected for the EQA3-WGS-AMR

Sample	EQA3-S24-01	EQA3-S24-02	EQA3-S24-03
Serotype	Rissen	Heidelberg	4,5,12:i:-
ST	469	15	34
Genes[A]	*aac(6')-Iaa, aph(6)-Id, aph(3'')-Ib, aac(3)-IIa, blaCTX-M-55, floR, qnrS1, sul2, tet(A)*	*aac(6')-Iaa, aadA1, aadA2, ant(3'')-Ia, blaCTX-M-123, blaTEM-1B, cmlA1, dfrA12, floR, fosA7, qnrS1, sul2, sul3*	*aac(6')-Ib, aac(6')-Ib3, aac(6')-IIc, aac(6')-Iaa, aadA2, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id, blaSHV-12, blaTEM-1B, dfrA19, ere(A), qnrB2, sul1, sul2, tet(B), tet(D)*
PMs[A]	gyrA p.D87N, parC p.T57S	parC p.T57S	None
NWT Phenotypes[B]	AMP, CTX, CAZ, CHL, CIP, GEN, NAL, TCY	AMP, AZM***, CTX, CAZ, CHL, CIP, TCY***, TMP	AMI, AMP, CTX, CAZ, CIP, GEN, NAL, TCY, TMP
NWT Predicted Phenotypes[C]	AMI, AMP, CEP*, CTX, CAZ, CHL, CIP, GEN, NAL, SMX**, TCY, TOB*	AMI, AMP, CEP*, CTX, CAZ, CHL, CIP, SMX**, TOB*, TMP	AMI, AMP, CEP*, CTX, CAZ, CIP, GEN, SMX**, TCY, TOB*, TMP

A According to ResFinder
B Abbreviations of antimicrobials: AMI (amikacin), AMP (ampicillin), AZM (azithromycin), CEP (cefepime), CTX (cefotaxime), CAZ (ceftazidime), CHL (chloramphenicol), CIP (ciprofloxacin), GEN (gentamicin), NAL (nalidixic acid), SMX (sulfamethoxazole), TCY (tetracycline), TMP (trimethoprim), TOB (tobramycin)
C All predicted phenotypes reported by ResFinder when the EQA3 protocol was followed
* Antimicrobial not tested by EQA provider
** No ECOFF available, not possible to compare to phenotypic result
*** No genetic determinant and / or predicted phenotype found

All three *Salmonella* samples harbour beta lactam genes such as *blaCTX-M-55*, *blaCTX-M-123* and *blaSHV-2,* which confer resistance to cephalosporins such as cefepime, cefotaxime and ceftazidime and *blaTEM-1B*, which confers resistance to ampicillin (2).

The presence of *sul1*, *sul2* and *sul3* genes confers sulfamethoxazole resistance and the *tet(A)*, *tet(B)* and *tet(D)* genes are responsible for resistance to tetracyclines (3). The *floR* gene, present in samples EQA3-S24-01 and EQA3-S24-02, as well as gene *clmA1* in the latter sample, coding for an efflux pump, are associated with chloramphenicol resistance (3)(4)(5). The *dfrA12* and *dfrA19* genes, responsible for trimethoprim resistance in samples EQA3-S24-02 and EQA3-S24-03, are associated with Class I or Class II integrons, plasmids or Salmonella Genomic Island 1 (SGI1) or SGI2 (6)(7).

Resistance to quinolones, such as ciprofloxacin and pefloxacin, in all three samples, is mediated by genes *qnrS1* and *qnrB2*, coding for a protein that protects the DNA gyrase from inhibition by this group of antimicrobials (7)(8)(9). This type of resistance is plasmid-mediated. All three samples have a number of aminoglycoside genes, including phosphotransferases (*aph*), acetyltransferases (*aac*) and nucleotidyl transferases (*aad* and *ant*), that modify and inactivate the aminoglycoside. Of these, the genes belonging to the two latter types can confer gentamicin resistance (3). Phenotypic resistance to aminoglycoside amikacin is observed in sequence EQA3-S24-03, which is likely mediated by gene *aac(6')-Ib3* (10).

The point mutation in *gyrA* (D87N) is responsible for nalidixic acid resistance (11). It has been shown that the presence of the *qnr* gene alone does not mediate resistance to nalidixic acid, as opposed to the presence of one or more point mutations (12).

The *ere(A)* gene in sample EQA3-S24-03 is responsible for resistance to macrolides such as erythromycin (2). However, the only macrolide present on the panel used for phenotypic testing of *Salmonella* by the EQA provider is azithromycin and a different gene, *mphA*, is associated with resistance to that macrolide (2). Therefore, we do not have the phenotypic or predicted confirmation of erythromycin resistance.

Of interest, in sample EQA3-S24-02, phenotypic resistance to azithromycin and tetracycline was observed based on MIC values, however, it was not among the predicted phenotypes in ResFinder. Likewise, the sample did not contain any known genes or PMs (such as *mphA* or mutations in *acrB* and *ramR* for azithromycin and *tet* genes for tetracycline (2)) that could indicate resistance to these two antimicrobials. The EQA provider was able to confirm the tetracycline NWT phenotype by detecting the *tetM* gene in this genome using a different tool, AMRFinderPlus. As for azithromycin, no AMR determinants were identified in AMRFinderPlus for this isolate either.

Table 2. Genotypic and phenotypic characteristics of the Campylobacter isolates selected for the EQA3-WGS-AMR

Isolate	**EQA3-C24-01**	**EQA3-C24-02**	**EQA3-C24-03**
Species	*C. coli*	*C. coli*	*C. jejuni*
ST	872	12073	7433
Genes[A]	*aadE-Cc, blaOXA-489, tet(O)*	*ant(6)-Ia, aph(3')-III, blaOXA-193, cat(pC194), erm(B), tet(O/32/O)*	*aph(2'')-If, aph(3')-III, blaOXA-193, cat, tet(O)*
PMs[A]	23S r.2075A>G, gyrA p.T86I	gyrA p.T86I, rpsL p.K43R	gyrA p.T86I
NWT Phenotypes[B]	CIP, ERY, TCY	CHL, CIP, ERY, TCY	CHL, CIP, GEN, TCY
NWT Predicted Phenotypes[C]	CIP, ERY, streptomycin*, TCY	CIP, ERY, streptomycin*, TCY	CIP, GEN, NAL*, TCY

A According to ResFinder
B Abbreviations of antimicrobials: CIP (Ciprofloxacin), ERY (Erythromycin), GEN (Gentamicin), STR (Streptomycin), TCY (Tetracycline)
C All predicted phenotypes reported by ResFinder when the EQA3 protocol was followed
* Antimicrobial not tested by EQA provider

In the *Campylobacter* samples, the ciprofloxacin (fluoroquinolone) resistance is mediated through the *gyrA* T86I point mutation (14), present in each isolate. The *23S* A2075G substitution in sample EQA3-C24-01 is responsible for erythromycin (macrolide)

resistance (14). Chloramphenicol (phenicol) resistance in samples EQA3-C24-02 and EQA3-C24-03 is due to the presence of *cat(pC194)* and *cat* genes, respectively (15). All samples harbour the *tet(O)* or *tet(O/32/O)* genes, which mediates tetracycline resistance. Gentamicin (aminoglycoside) NWT phenotype identified in sample EQA3-C24-03 is likely due to the presence of *aph* genes (16)(17)(18).

The prediction of phenotypes in ResFinder indicates, additionally, resistance to streptomycin (aminoglycoside) in samples EQA3-C24-01 and EQA3-C24-02, caused by the presence of *aadE-Cc* and *ant(6)-Ia* genes, respectively (17). The *rpsL* K43R substitution in sample EQA3-C24-02 contributes additionally to streptomycin resistance (19); however, streptomycin resistance was not confirmed phenotypically in this EQA due to absence of this antimicrobial on the panels used by the EQA provider (see paragraph 2.1 for details). Resistance to nalidixic acid (quinolone) was predicted in sample EQA3-C24-03, but not confirmed phenotypically, due to the same reason as above. Resistance to this antimicrobial is likely mediated through the *gyrA* T86I substitution (14).

Two different variants of the *blaOXA* genes are present in all samples, without giving rise to laboratory-based or predicted phenotype. The expected phenotype would be resistance to beta-lactam antibiotics, which are not present on the panels used for testing by the EQA provider. In general, it is complicated to correlate the presence of *blaOXA* genes in *Campylobacter* spp. to a phenotype. Recent literature suggests that it is the presence of a G to T mutation in the promoter region of the *blaOXA-61* gene that is responsible for conferring resistance to ampicillin and not the presence of the gene alone (17)(20)(21).

2.3. Isolate culturing, DNA extraction and distribution

The DNA samples were prepared by DTU. The isolates were cultured on blood agar plates. The *Salmonella* isolates were incubated aerobically for 16-20 hours at 35±1°C and the *Campylobacter* isolates were incubated for 24 hours at 41.5±1°C in microaerophilic atmosphere.

After initial incubation, a lawn of bacteria was created on blood agar plates by streaking a suspension of the isolates using a plate rotator, followed by overnight incubation. Colony mass was harvested with a 10 µl loop from each isolate and resuspended in 3 ml PBS. Tubes were centrifuged at 20.000 G for 5 minutes and after decantation, the pellet was resuspended in 600 µl PBS buffer.

For DNA isolation, the Easy-DNA Kit for genomic DNA isolation was used (Invitrogen). Protocol #3 from the kit was applied in triple volume and nuclease-free water with 2 mg/ml RNase was used to resuspend the precipitated pellet. The DNA concentration was measured using Qubit with 2 µl DNA sample. The DNA was diluted to approximately 50 ng/µl and stored at -20°C.

Twenty microliter of the dilution was aliquoted into 1.5 ml Eppendorf tubes and vaccum dried using a vacuum centrifuge (Eppendorf Concentator plus) for 30 minutes at temperature 25-30°C using the default settings. The tubes with dried samples were stored in bags at room temperature together with a silica bag. The pellet from one tube of each strain was resuspended in 50 µl nuclease free water and concentration was measured using Qubit™ dsDNA Quantification High Sensitivity (HS) kit. The measured concentrations are presented in Table 3.

Table 3. DNA concentrations measured using Qubit™ 1X dsDNA HS kit

Species	Strain	DNA amount per tube [ng]
Salmonella	EQA3-S24-01	3016.7
	EQA3-S24-02	2883.3
	EQA3-S24-03	2150
Campylobacter	EQA3-C24-01	5393.3
	EQA3-C24-02	6006.7
	EQA3-C24-03	2956.7

Forty-five tubes with dried DNA from each strain were produced and delivered to SSI, packed in zip-lock bags together with silica bags placed in bubble envelopes. The packages with DNA samples were shipped at room temperature.

2.4. EQA3 protocol

The protocol with detailed instructions on how to analyse the EQA3-WGS-AMR samples and report the results (see Section 9.1.1) was distributed to all participants by e-mail and placed on the FWD AMR-RefLabCap website (to be found under https://www.fwdamr-reflabcap.eu/eqas-and-ring-trials/eqas). In order to be able to compare the results between laboratories, all participants were asked to follow this protocol that also provided a framework for uniform reporting of the applied methods, as well as the identified genes and PMs. All participants were asked to upload the same type of file (fastq), use the same database and software version and to apply the same way to report the genes and PMs. This ensured that the possible differences among the genes reported by the participants would be due to issues related to sequencing or DNA quality in the participating laboratories.

When an analysis is finished in ResFinder, the results are available on a website, as well as in multiple text files that are available for download at the bottom of the result website. In the EQA3 protocol, it is specified by the use of screenshots, which of the files should use to report the result. The idea was to report all genes or PMs listed in the specific text files, without the need for the participant to decide whether the gene is relevant or not.

2.5. WGS analysis by the EQA provider

DNA from *Salmonella* and *Campylobacter* isolates was sequenced using paired-end Illumina sequencing. The quality of the sequences (genome size, N50, total number of contigs) was checked with an in-house QC pipeline (https://github.com/ssi-dk/bifrost) for raw reads and BioNumerics for assemblies.

Salmonella serotypes were determined using Enterobase and SeqSero (https://github.com/denglab/SeqSero), as well as in-house developed scripts detecting the subspecies and genetic marker implicating the *d*-Tartrate reaction for distinguishing *S* Paratyphi B var. L(+) tartrate+ (var. Java) from *S* Paratyphi B.

For *Campylobacter* species identification, Kraken was used (https://github.com/DerrickWood/kraken). MLST calling was performed with ARIBA (https://github.com/sanger-pathogens/ariba) using the typing schemes from the PubMLST database.

The reads were analysed by the EQA provider for the presence of AMR genes and PMs by querying the ResFinder database using the online ResFinder tool, version 4.4.2 (http://genepi.food.dtu.dk/resfinder) according to the EQA3 protocol (Section 9.1.1), shared with the participants.

All participants were asked to upload the reads produced in their laboratory to an ftp site. The reads were analysed using the same QC pipeline as the EQA provider (https://github.com/ssi-dk/bifrost).

The quality of the generated sequences was evaluated by EQA provider against the thresholds recommended in the suggested WGS protocol (https://www.fwdamr-reflabcap.eu/resources/reflabcap-protocols-and-guidelines) which are a genome size of 4.4 Mb-5.8 Mb for *Salmonella*, and 1.5 Mb - 1.9 Mb for *Campylobacter*, a N50 of > 30 000 bp, a contig number of <500, and a coverage of minimum 30x.

2.6. SurveyXact reporting scheme and collection of results

The reporting platform was developed in the SurveyXact survey tool (https://rambollxact.com).

The reporting scheme consisted of two parts. The first part included questions about sequencing technology, method for DNA concentration measurement, DNA concentrations, as well as tools used for identifying sequence type (ST), serotype (for *Salmonella*) and species (for *Campylobacter*). The second part was for reporting AMR genes, point mutations (PMs) and predicted phenotypes. It was possible to select multiple genes from a list in alphabetical order, as well as report genes in a free text field, in case it was not present on the default list. For reporting of point mutations, the participants were asked to type the detected mutations in text boxes. Reporting of the predicted phenotypes consisted of marking “WT”, “NWT” or “Not determined” for a defined list of antimicrobials, to which the EQA provider has determined phenotypes in the laboratory.

All participants received individual links to the reporting form, where it was possible to report results for one or both pathogens. The time given for reporting of the results was two months from the sample shipping date.

Twenty-six laboratories reported for *Salmonella* (including nineteen laboratories reporting for both *Salmonella* and *Campylobacter* and seven for *Salmonella* only). Twenty-four laboratories reported for *Campylobacter* (including nineteen reporting for both *Salmonella* and *Campylobacter* and five for *Campylobacter* only). The participating countries were Austria, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Greece, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Moldova, the Netherlands, Portugal, Slovenia, Spain and Sweden. The participating laboratories were randomly assigned codes and these codes were used for identification of laboratories in the EQA.

3. Salmonella results

3.1. Quality metrics for all Salmonella DNA samples

3.1.1. DNA measurement evaluation

All 26 participants reported DNA concentration results and the methods used for concentration determination for all three *Salmonella* samples (Figure 1, Table S1). Of the 78 reported results for all three samples, 60 (77%) were between 10.4 and 40.9 ng/µl, five results, reported by four participants, were below 10 ng/µl and 13 results reported by seven participants were above 50 ng/µl (Figure 1).

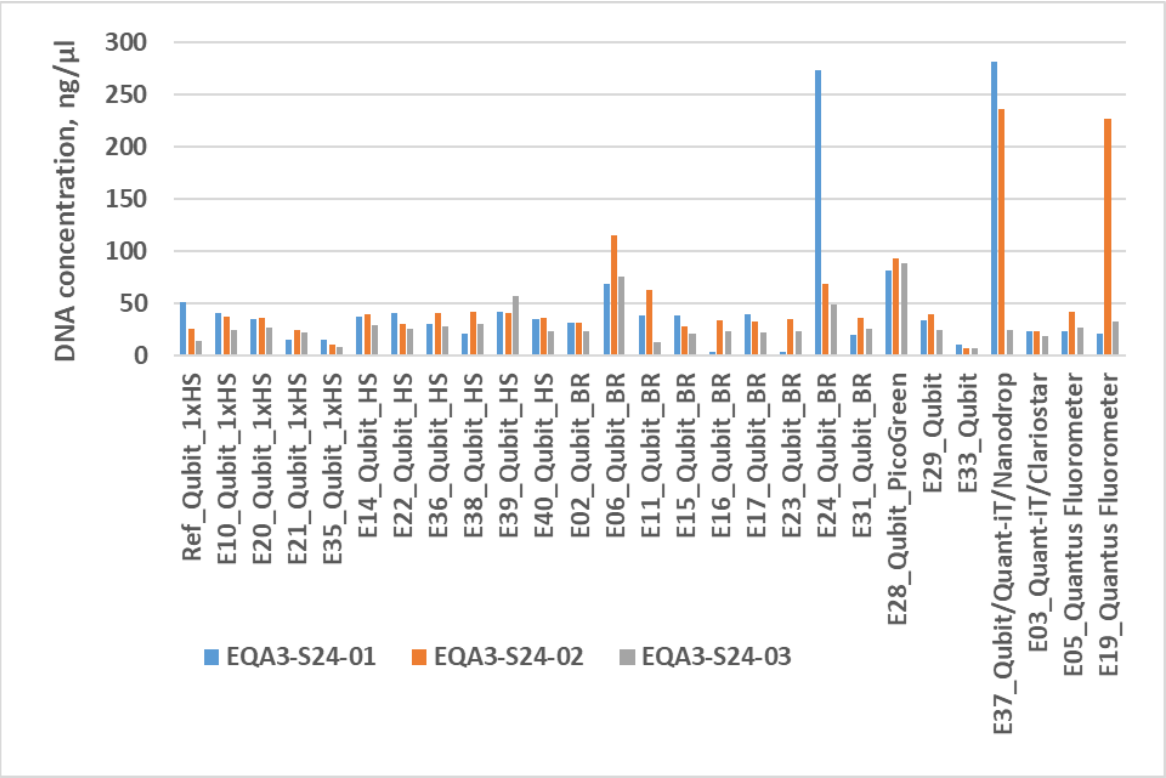

Figure 1. The distribution of Salmonella DNA concentrations among 26 participants with the indicated method(s) used.

To measure DNA concentration, most participants (n=11) used either Qubit High Sensitivity (HS) or 1x High Sensitivity kits (1xHS). Qubit Broad Range kit (BR) kit was used by nine participants. Remaining participants (n=7) did not specify the kit used or used other kits.

Lower variation in DNA measurements was reported when Qubit 1x HS (M=26, SD=12) or HS (M=35, SD=9) kits were in use, and much higher when Qubit BR (M=46, SD=51) and other instruments or kits (M=65, SD=81) were in use (Figure 2).

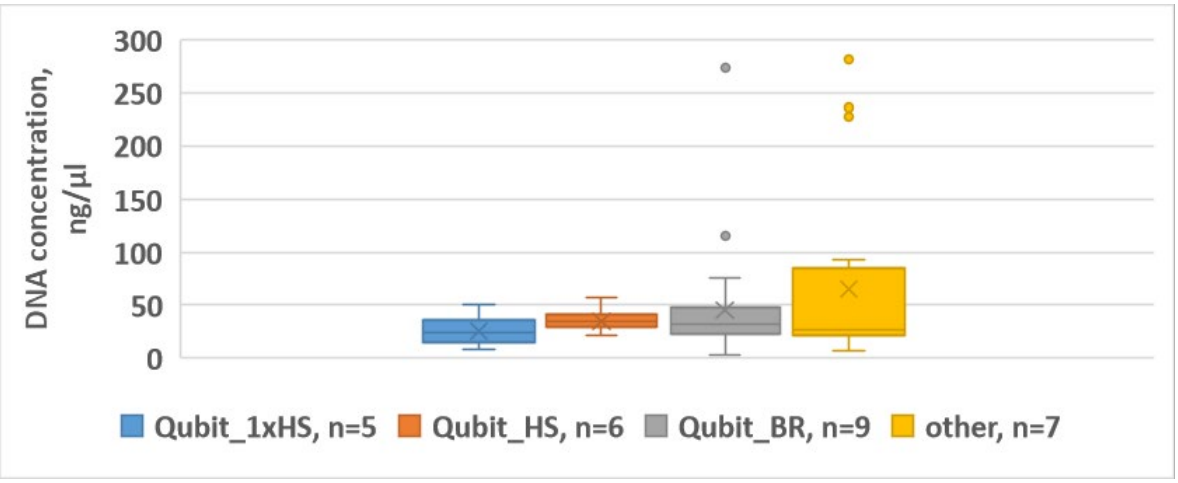

Figure 2. The distribution of Salmonella DNA concentrations among 26 participants when different DNA measurement methods are in use. The number at each category indicates the number of participants that used a specific method (EQA provider is included in category Qubit_1xHS). All categories include measurements of all three Salmonella strains.

3.1.2. Sequencing quality evaluation

Twenty-three participants used Illumina technology, two participants used Ion Torrent technology and one participant used Nanopore technology. Among the 23 participants using Illumina technology, 12 used DNA Prep library preparation kit, eight used Nextera XT kit, one used DeepChek® NGS Library Preparation Kit, and two did not provide information on the kit they used. The participants using Ion Torrent technology applied different sequencing library preparation kits. The participant that used nanopore technology applied the Rapid kit 96 V14 (Figure 3).

The sequence quality analysis revealed that all three sequences generated by 21 (80%) participants passed the defined quality thresholds for all parameters.

Three participants (E06, E33, E37) submitted sequences of which two passed the QC evaluation, participant E35 submitted one sequence that passed the QC evaluation and none of the sequences from E05 passed the QC evaluation (Tables S3 – S5).

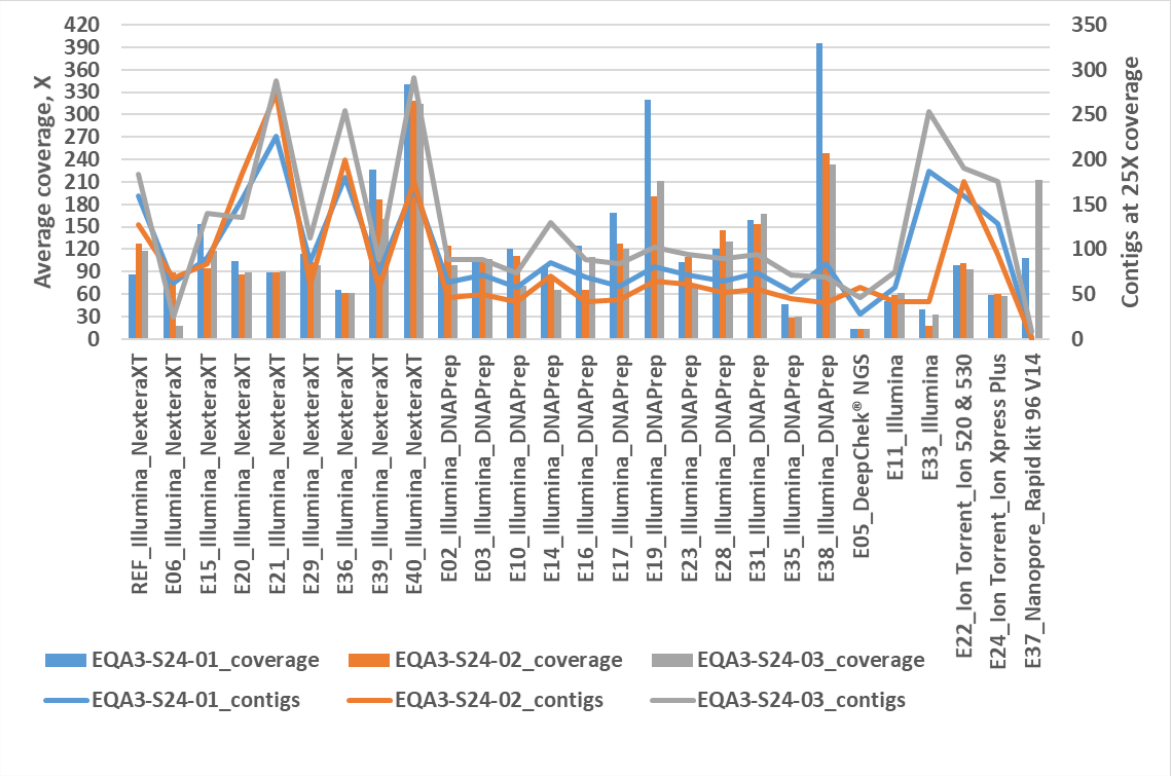

Figure 3. The distribution of average sequence coverage and number of contigs for Salmonella among 26 participants with the indicated sequencing technology and library preparation kit used.

Sequencing with the Illumina DNA Prep library preparation kit provided a less variable number of contigs at 25X (M=70, SD=20) compared to sequencing using Illumina Nextera XT kit (M=146, SD=75) and other sequencing technologies and kits (M=101, SD=76), independently from sequencing coverage for all participants (Figure 4).

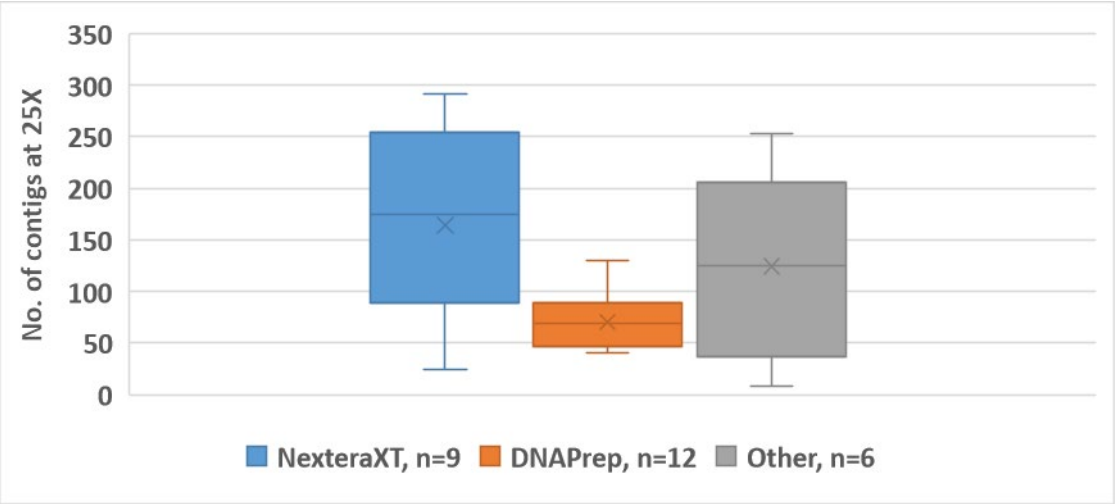

Figure 4. The distribution of number of contigs among 26 participants when different sequencing library preparation kits are in use. The number at each category indicates the number of participants that used a specific sequencing library preparation kit (EQA provider is included in category Nextera_XT). All categories include number of contigs for all three Salmonella strains.

3.2. Serotypes and STs reported

3.2.1. Serotyping methods and serotypes

Eighteen participants used one tool for *Salmonella* serotyping and eight participants used a combination of two tools. The most commonly used tool was SeqSero, that was used by 24 participants (Figure 5).

The most commonly reported versions of SeqSero were SeqSero 1.2 and SeqSero2 v1.2.1, that were used by 10 and seven participants, respectively. The remaining seven participants indicated other SeqSero versions or did not provide details.

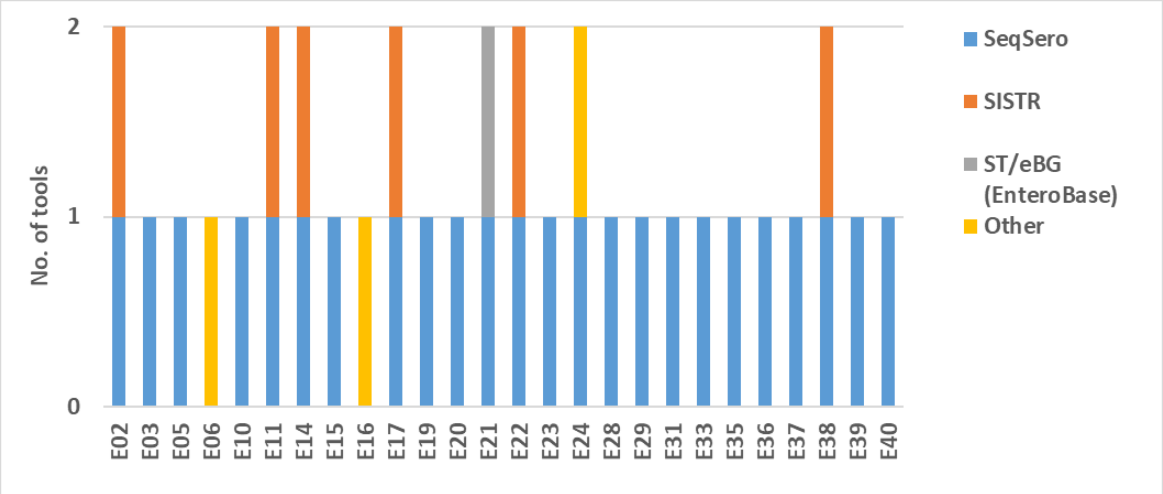

Figure 5. An overview of the tools used by 26 participants for Salmonella serotyping

All 26 participants reported serotypes for sample EQA3-S24-01 and 25 participants reported serotypes for samples EQA3-S24-02 and EQA3-S24-03 (Table 4). Participant E37 did not report any data for the two latter samples due to insufficient DNA quality for sequencing. The serotype reported by this participant for samples EQA3-S24-01 was incorrect (Table S9). The remaining 25 participants reported all three serotypes in concordance with the provider's results (Table 4).

Table 4. Reported Salmonella serotypes and concordance with EQA provider's results

Sample	EQA3-S24-01	EQA3-S24-02	EQA3-S24-03
Serotype	Rissen	Heidelberg	4,5,12:i:-
No. of concording results	25	25	25
No. of non-concording results	1	0	0
No. of missing serotype	0	1	1

3.2.2. MLST methods and STs

Ten participants used the Tsemann MLST scheme, nine participants used the MLST2.0 scheme available from CGE tools (https://cge.food.dtu.dk/services/MLST/), six participants used SeqSphere and four participants used Enterobase. One participant used BioNumerics in addition to SeqSphere (Table S10).

Twenty-five out of 26 participants reported ST for isolates EQA3-S24-01 (ST469) and EQA3-S24-03 (ST34), and 24 participants reported ST for sample EQA3-S24-02 (ST15). All reported STs were in agreement with the EQA provider's results.

3.3. AMR genes and PMs reported for *Salmonella* DNA samples

The genes and PMs identified by the EQA provider using ResFinder (according to the EQA3 protocol, Section 9.1.1) and the genes identified by the participants are presented for all three samples in the following paragraphs.

For each gene and PM table, the concordance of the reported results among the participants was calculated as the percentage of the total number of participants that reported the same genes or PMs for a given DNA sample. If a participant deemed the quality of DNA to be insufficient for reporting genes or PMs for a given sample, the participant was not included in the calculation for that sample.

When possible, explanations of observed discrepancies between the reference dataset and participants' results, or in between the participants, are provided.

In this EQA3-WGS-AMR, the EQA provider expected similar results from all participants, as the participants were asked to follow the same overall protocol (Section 9.1.1). The participants were expected to:

- Use the same type of input file for the analysis (fastq reads)
- Use the same online tool (ResFinder) and the same version (4.4.2)
- Apply the same settings, such as identity and coverage cut-off thresholds, selected species etc., as specified in the EQA3 protocol, when submitting the reads
- Follow the instruction on how to report the obtained findings (by downloading the specified text files and reporting all the genes and PMs listed instead of reporting the output on the website)

3.3.1. Genes reported in *Salmonella* samples

The table below (Table 5) includes only genes that were reported in the reference dataset and by the participants. All additional genes reported by the participants, but not in the reference dataset, can be found in supplementary tables (Annex C).

Table 5. Genes reported in Salmonella samples EQA3-S24-01, EQA3-S24-02 and EQA3-S24-03. Reference dataset, Ref, is shaded grey. Participants are presented in numerical order. The letter "X" indicates the reported gene. Percentage concordance (%C) is based on the following scale: darkest orange colour: 100% concordance among participants, lighter

orange colour: 90-99% concordance, lightest orange colour: 80-89% concordance. Concordance lower than 80% is without colour.

EQA3-S24-01																												
	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40	%C
aac(3)-IIa	X		X	X		X		X	X	X	X	X	X	X	X	X	X	X	X	X		X	X		X		X	77
aac(6')-Iaa	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	X		X	X	X	X	X	X	88
aph(3'')-Ib	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	92
aph(6)-Id	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X		X	X	X	92
blaCTX-M-55	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	100
floR	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	96
qnrS1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	100
sul2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	100
tet(A)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	100
EQA3-S24-02																												
	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40	%C
aac(6')-Iaa	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	X	X	X	X		X	X	X	92
aadA1	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	92
aadA2	X	X	X		X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X		X	X	X	92
ant(3'')-Ia	X							X		X		X		X				X				X					X	28
blaCTX-M-123	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
blaTEM-1B	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
cmlA1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
dfrA12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
floR	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
fosA7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
qnrS1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
sul2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
sul3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	96
EQA3-S24-03																												
	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40	%C
aac(6')-Ib	X																		X	X					X			12
aac(6')-Ib3	X	X	X		X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	88
aac(6')-IIc	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
aac(6')-Iaa	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	X	X	X	X		X	X	X	92
aadA2	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	96
aph(3')-Ia	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
aph(3'')-Ib	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	96
aph(6)-Id	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	96
blaSHV-12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
blaTEM-1B	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
dfrA19	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
ere(A)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X		X	X	X	96
qnrB2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
sul1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
sul2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	96
tet(B)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
tet(D)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100

Participant E37 did not perform analysis of samples EQA3-S24-02 and EQA3-S24-03 due to insufficient DNA quality.

In general, the concordance was good, as 87% of genes in all three samples were reported by more than 90% of participants. However, it is evident that some participants did not strictly adhere to the EQA3 protocol and applied their own criteria for reporting genes and this is likely the main reason for the observed differences between the EQA provider and participants' results.

Gene *aac(3)-IId* was reported in sample EQA3-S24-01 by 11 out of 26 participants (Table S10), but was not reported by the EQA provider. Participant E28 noted that this gene was found only in contigs and not in fastq files, indicating that they used assemblies for data analysis in addition to reads, which was not the intention of the protocol. Another additional gene (*aadA2b*), found using assembly, was reported by participant E28 in sample EQA3-S24-03 (Table S12).

Only one participant (E05) did not report the *floR* gene in sample EQA3-S24-01, but reported that the gene was detected with 96.46% identity. Similarly, in sample EQA3-S24-02, identity of 98.66% for the *aadA2* gene was the reason for the same participant not to report that gene. In sample EQA3-S24-03, genes *aadA2* and *aac(6')-Ib* were detected, but not reported by the same participant, due to identities of 99.51% and 94.39%, respectively. The EQA3 protocol, however, specified that all genes with more than 90% identity and 60%

coverage should be reported (all genes summarized in the file that was to be downloaded – see points 14 to 15 in the EQA3 protocol (Section 9.1.1)).

In sample EQA3-S24-02, gene *tetM* was reported by 5 out of 26 participants (Table S11), but not by the EQA provider. Participant E28 noted that this gene was found only when assemblies were used. This was confirmed by the EQA provider when assemblies were used in AMRFinderPlus. In ResFinder (the version recommended by the EQA3 protocol), lowering the identity and coverage thresholds to 30% and 20%, resulted in four hits of the *tet(M)* gene with identities between 32 and 62%. This indicates that the mapping method used by ResFinder could not map the whole gene to a specific variant in ResFinder database.

In sample EQA3-S24-02, only 7 out of 26 participants reported the *ant(3'')-Ia* gene, as did the EQA provider. It is known that the *ant(3'')-Ia* gene is an alternative name for the *aadA2* gene and this could the reason why 19 participants did not report this gene.

Gene *aac(6')-Ib*, reported in the reference dataset (90.92% identity, 92.08% coverage), was only detected by 3 participants. One of them, E29, noted that the gene was detected with 90.26% identity, similar to the one from the reference dataset, but also very close to the identity cut-off of 90%, set in the EQA3 protocol. The quality of sequencing could have affected the detection of this gene, if it led to identity below 90%. However, participant E05 did not report this gene due to an identity of 94.39%, which was deemed too low by this participant. This supports the theory that some participants might have used their own cut-offs for reporting, instead of the ones specified in the EQA3 protocol.

3.3.2. Point mutations reported in *Salmonella* samples

Point mutations reported in reference dataset in samples EQA3-S24-01 and EQA3-S24-02 are presented in Table 6. Additional point mutations reported by the participants, but not by the EQA provider, can be found in supplementary tables (Annex C).

Table 6. Point mutations (PMs) reported in Salmonella samples EQA3-S24-01 and EQA3-S24-02. Reference dataset, Ref, is shaded grey. Participants are presented in numerical order. The letter "X" indicates the reported PM. Percentage concordance (%C) is based on the following scale: darkest orange colour: 100% concordance among participants, lighter orange colour: 90-99% concordance, lightest orange colour: 80-89% concordance. Concordance lower than 80% is without colour.

EQA3-S24-01																												
	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40	%C
parC T57S	X	X	X	X	X	X		X	X	X	X		X	X	X	X	X	X	X	X	X	X	X			X	X	88
gyrA D87N	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
EQA3-S24-02																												
	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40	%C
parC T57S	X	X	X	X	X	X		X	X	X	X	X		X	X	X	X	X	X	X	X	X	X			X	X	88

Participant E37 did not perform analysis for point mutations of samples EQA3-S24-01 and EQA3-S24-02 due to insufficient DNA quality.
No point mutations were reported in the reference dataset nor by the participants in EQA3-S24-03.

The substitution D87N in *gyrA* in sample EQA3-S24-01 was reported by all participants that estimated the DNA quality to be sufficient. The mutation *parC* T57S was reported by 88% of participants. The reason for three participants not reporting this mutation can be the lack of consensus on whether this PM contributes to quinolone resistance in *Salmonella* (22), but it would be against the instructions in the EQA3 protocol.

3.4. Predicted phenotypes for *Salmonella* samples

The tables below show the predicted phenotypes reported by the EQA provider and the participants in the three *Salmonella* samples. According to the EQA3 protocol (Section 9.1.1), the participants were asked to report "the Resistant WGS-predicted phenotype for the antimicrobials included in the predicted phenotype question in the reporting scheme." The options in the reporting scheme included "wild type" (WT - sensitive), "non-wild type"

(NWT – resistant) and "not determined" (ND). The latter option could be used if ResFinder did not give a WT or NWT prediction.

In the tables below, the WT and NWT phenotype predictions that diverged from the EQA provider's reported predictions, are marked with a blue colour. The ND option, which diverges from the EQA provider's prediction, is marked in yellow.

Table 7. Predicted phenotypes in sample EQA3-S24-01. WT – wild type, NWT – non wild type, ND – not determined. Blue colour: WT and NWT phenotype predictions diverging from the EQA provider's reported predictions. Yellow colour: ND diverging from the EQA provider's prediction.

	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40
Amikacin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	WT	NWT	NWT	NWT	WT	ND	NWT	NWT	NWT		NWT	NWT
Ampicillin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	WT		NWT	NWT
Azithromycin	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Cefotaxime	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Ceftazidime	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Chloramphenicol	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Ciprofloxacin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	WT		NWT	NWT
Colistin	WT	WT	WT	WT	WT		WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Gentamicin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	NWT	NWT	WT	NWT	NWT	NWT		NWT	NWT
Meropenem	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Nalidixic acid	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	ND	WT	WT	NWT	WT		NWT	NWT
Sulfamethoxazole	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	WT	WT		NWT	NWT
Tetracycline	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	WT		NWT	NWT
Tigecycline	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT	WT	NWT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Trimethoprim	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT

Participant E38 did not detect any phenotypes

Table 8. Predicted phenotypes in sample EQA3-S24-02. WT – wild type, NWT – non wild type, ND – not determined. Blue colour: WT and NWT phenotype predictions diverging from the EQA provider's reported predictions. Yellow colour: ND diverging from the EQA provider's prediction.

	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40
Amikacin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	WT	NWT	NWT	NWT	WT	WT	NWT	NWT			NWT	NWT
Ampicillin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Azithromycin	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Cefotaxime	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Ceftazidime	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Chloramphenicol	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Ciprofloxacin	NWT	NWT	NWT	NWT	WT	NWT	WT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Colistin	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Gentamicin	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NWT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Meropenem	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Nalidixic acid	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NWT	NWT	ND	WT	NWT	WT	NWT	WT		NWT	WT	WT			WT	WT
Sulfamethoxazole	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Tetracycline	WT	NWT	WT	WT	NWT	WT	NWT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	NWT	WT		NWT	WT	WT			WT	WT
Tigecycline	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Trimethoprim	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT

Participant E37 and E38 did not detect any phenotypes

Table 9. Predicted phenotypes in sample EQA3-S24-03. WT – wild type, NWT – non wild type, ND – not determined. Blue colour: WT and NWT phenotype predictions diverging from the EQA provider's reported predictions. Yellow colour: ND diverging from the EQA provider's prediction.

	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40
Amikacin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	WT	NWT	NWT	NWT	WT	WT	NWT	NWT			NWT	NWT
Ampicillin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Azithromycin	WT	WT	WT	WT	WT		WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	NWT	WT		NWT	WT	WT			WT	WT
Cefotaxime	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Ceftazidime	NWT	NWT	NWT	NWT	NWT		NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Chloramphenicol	WT	WT	WT	WT	WT		WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Ciprofloxacin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Colistin	WT	WT	WT	WT	WT		WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Gentamicin	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Meropenem	WT	WT	WT	WT	WT		WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT	WT			WT	WT
Nalidixic acid	WT	WT	WT	WT	NWT		NWT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	WT				WT	WT
Sulfamethoxazole	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Tetracycline	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT
Tigecycline	WT	WT	WT	WT	ND		WT	WT	WT	WT	WT	WT	WT	ND	WT	WT	WT	WT	WT		NWT	NWT	WT			WT	WT
Trimethoprim	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT			NWT	NWT

Participant E37 and E38 did not detect any phenotypes

For the *Salmonella* samples, one participant reported consistently divergent results, compared to the reference dataset. A possible explanation for this could be that participant E33 swapped the abbreviations "WT" and "NWT". In the ResFinder predicted phenotype

report, the results are reported as “Resistant” or “No resistance”. Apart from this participant, there were only a few diverging results.

4. *Campylobacter* results

4.1. Quality metrics for all *Campylobacter* strains

4.1.1. DNA measurement evaluation

Overall, 23 out of 24 participants reported DNA concentrations and the methods used for concentration determination in the three *Campylobacter* samples (Figure 6, Table S2). One participant, E12, did not report information about the methods and the concentration, due to “outsourcing of NGS”. Most reported DNA concentrations (n=52, 75%) were in the range from 11.9 to 85.2 ng/µl.

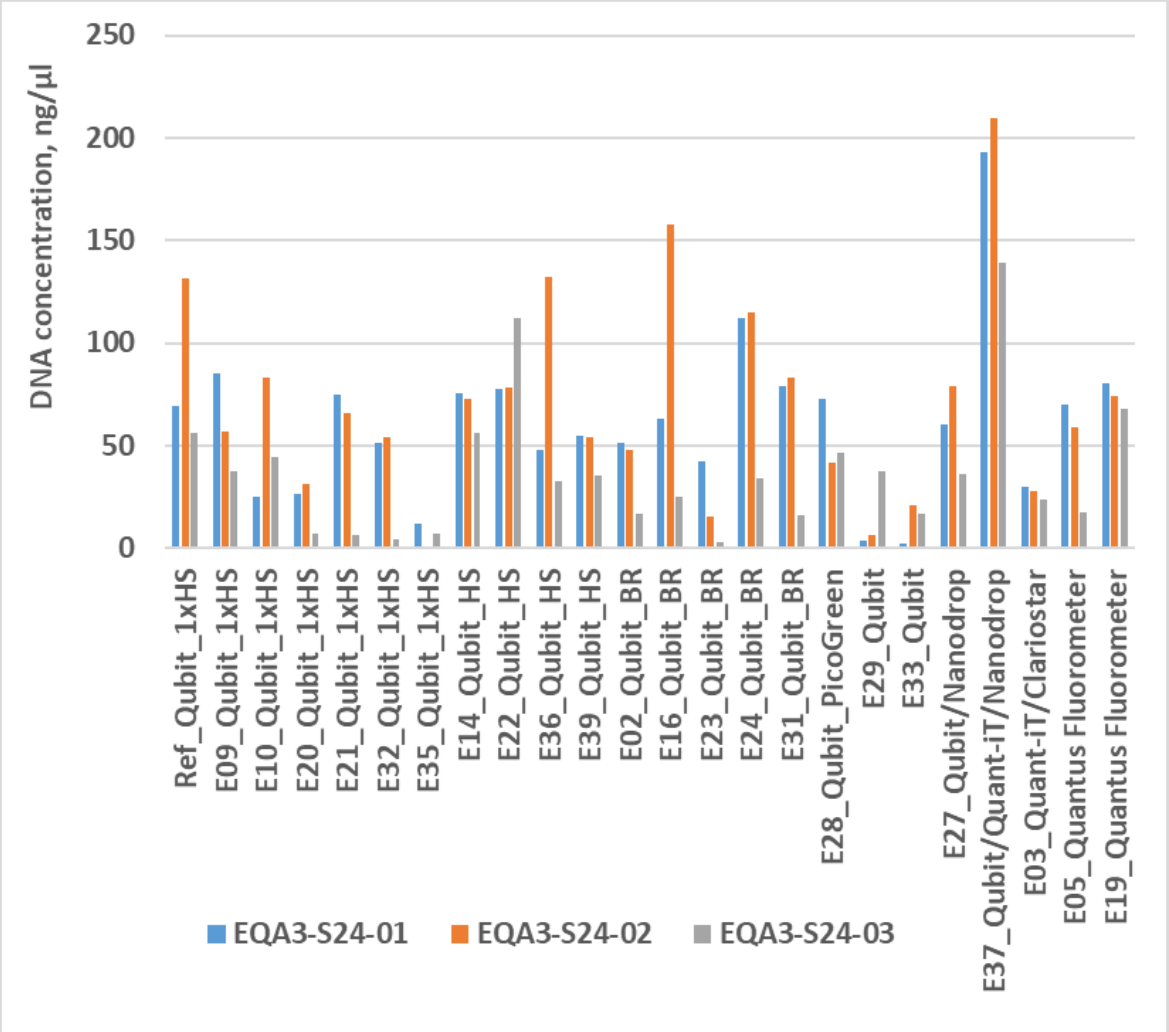

Figure 6. The distribution of Campylobacter DNA concentrations among 23 participants with the indicated method(s) used.

To measure DNA concentration, most participants (n=11) used either Qubit High Sensitivity (HS) or Qubit 1x High Sensitivity kits (1xHS). Qubit Broad Range kit (BR) kit was used by five participants. Remaining participants (n=8) either did not specify the kit used or used other kits.

The lowest variation in DNA measurements was reported when Qubit dsDNA high sensitivity HS (M=69, SD=29) kit was used. Higher variation in measurements was reported

for other kits: 1x HS (M=44, SD=34), Qubit dsDNA broad range (BR) (M=57, SD=44) and other instruments or kits (M=59, SD=54) (Figure 7).

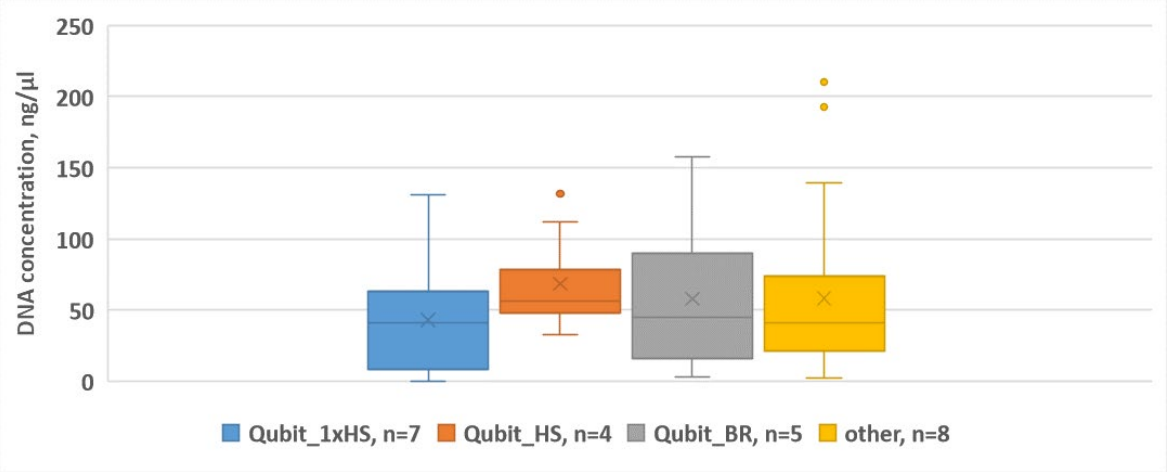

Figure 7. The distribution of Campylobacter DNA concentrations among 23 participants when different DNA measurement methods are in use. The number at each category indicates the number of participants that used a specific method (EQA provider is included in category Qubit_1xHS). All categories include measurements of all three Campylobacter strains.

4.1.2. Sequencing quality evaluation

Twenty-two participants used Illumina technology, one participant used Ion Torrent technology and one participant used Nanopore technology. Among the participants that used Illumina technology, 12 applied DNA Prep sequencing library preparation kit, seven used Nextera XT kit, one used DeepChek® NGS Library Preparation Kit, and two did not provide information on the kit used (Figure 8).

The sequence quality analysis performed by the EQA provider revealed that the sequences generated by 17 (70%) of the participants passed quality thresholds for all parameters.

The sequences generated for sample EQA3-C24-01 did not pass the N50 threshold for participants E05, E21, E24, and E36. For participant E33, both N50 and the average coverage thresholds were not passed. The sequences generated by participant E33 for sample EQA3-C24-02 did not pass the requirements for genome length, average coverage and N50, and for participant E21 the N50 threshold was not met. Finally, the sequences generated for sample EQA3-C24-03 did not pass the N50 threshold for participants E20, E21, E33 and E36, and genome length and average coverage thresholds for participant E37 (Table S8).

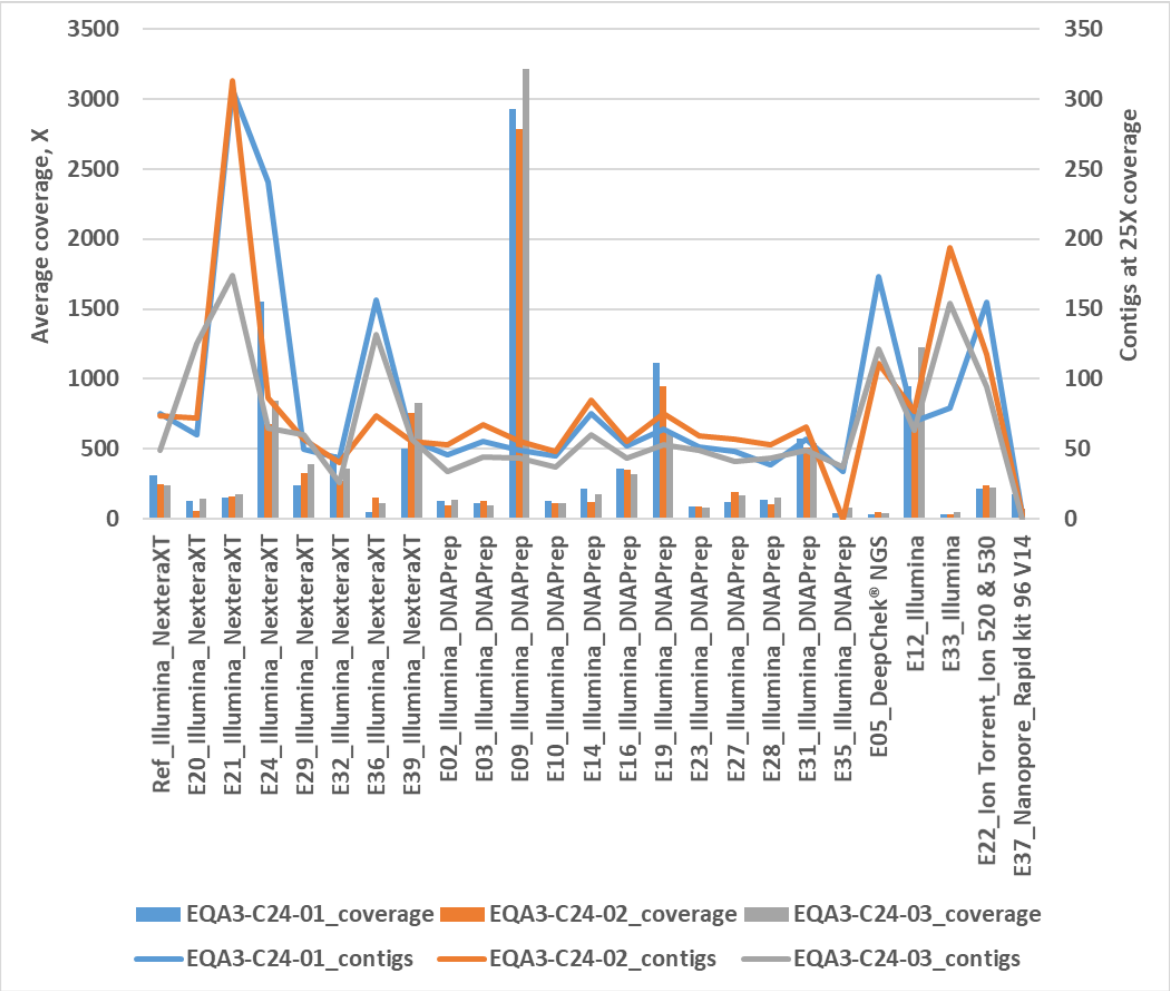

Figure 8. The distribution of average sequence coverage and number of contigs among 24 Campylobacter participants with the indicated sequencing technology and library preparation kit used.

Similarly to Salmonella samples, sequencing using Illumina DNA Prep library preparation kit provided a less variable number of contigs at 25X (M=53, SD=11) compared to sequencing using Illumina Nextera XT kit (M=102, SD=79) and other sequencing technologies and kits (M=87, SD=59), independently from sequencing coverage for all participants (Figure 9).

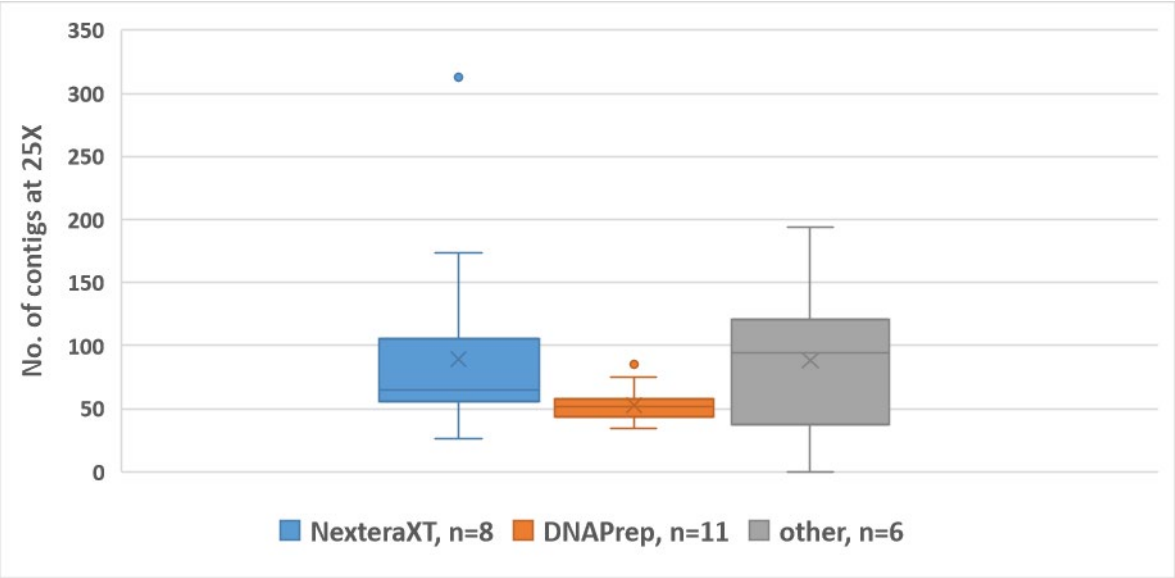

Figure 9. The distribution of number of contigs among 24 participants when different sequencing library preparation kits are in use. The number at each category indicates the number of participants that used a specific sequencing library preparation kit (EQA provider is included in category Nextera_XT). All categories include number of contigs for all three Campylobacter strains.

4.2. Species and STs reported

4.2.1. Species identification: methods and reported results

Nineteen participants used one tool for *Campylobacter* species detection and five participants used two tools. The most commonly used tool was KmerFinder, used by 11 participants. The remaining tools are shown in Figure 10.

Almost all participants reported the correct *Campylobacter* species for all three samples (data not shown). Participant E35 did not report species for strain EQA3-C24-02 due to QC fail and reported incorrect species for strain EQA3-C24-03. Participant E37 reported incorrect species for all three samples.

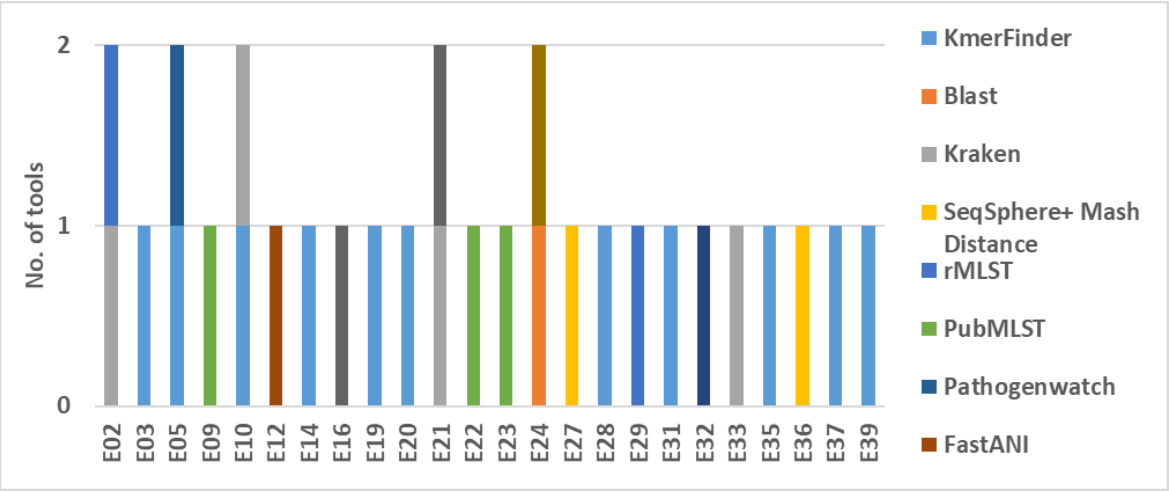

Figure 10. An overview of the tools/software used by 24 participants for Campylobacter species identification.

4.2.2. MLST methods and STs

The most commonly used methods for 7-gene MLST of *Campylobacter* were the MLST2.0 scheme available through CGE tools and the tsemann MLST scheme, used by

nine and seven participants, respectively. Six other methods were applied by up to four participants (Figure 11).

Twenty-three out of 24 participants reported the correct ST for *Campylobacter* samples EQA3-C24-01 (ST 872) and EQA3-C24-03 (ST 7433) (data not shown). One participant (E37) did not report ST for any samples. Of twenty participants that reported the ST for sample EQA3-C24-02 (ST 12073), nineteen reported the correct ST, one participant (E16) reported an incorrect ST (830) and four did not report the ST. Participant E35 reported QC fail for this sample (Table S7).

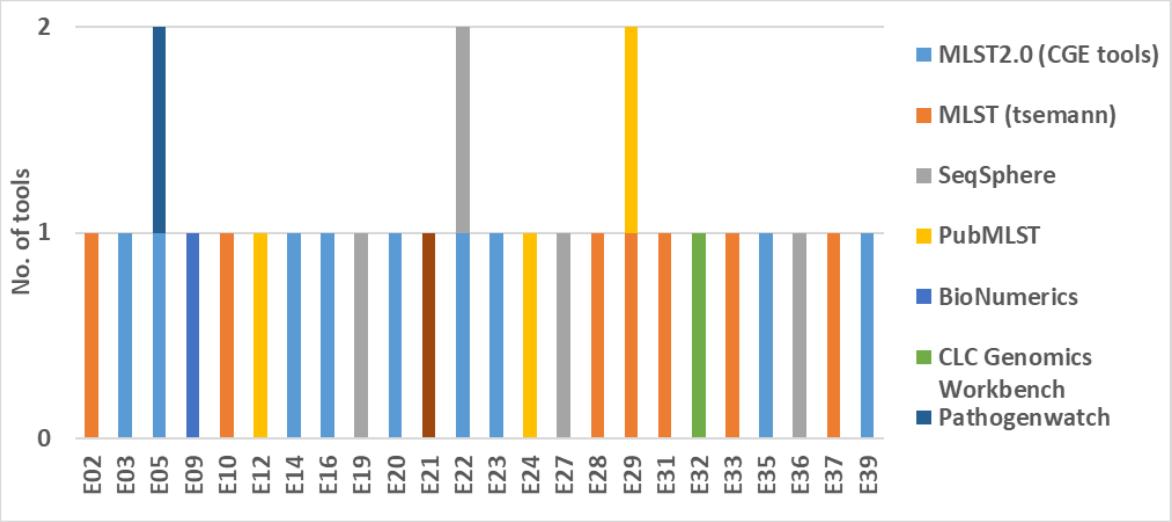

Figure 11. An overview of the tools/softwares used by 24 participants for Campylobacter 7-gene MLST.

4.3. AMR genes and PMs reported for *Campylobacter* strains

The genes and PMs identified by the EQA provider using ResFinder (according to the EQA3 protocol, Section 9.1.1) and the genes identified by the participants are presented for all three samples in the following paragraphs.

For each gene and PM table, the concordance of the reported results among the participants was calculated as the percentage of the total number of participants that reported the same genes or PMs for a given DNA sample. If a participant deemed the quality of DNA to be insufficient for reporting genes or PMs for a given sample, the participant was not included in the calculation for that sample.

When possible, explanations of observed discrepancies between the reference dataset and participants' results, or in between the participants, are provided.

In this EQA3-WGS-AMR, the EQA provider expected similar results from all participants, as the participants were asked to follow the same overall protocol (Section 9.1.1). The participants were expected to:

- Use the same type of input file for the analysis (fastq reads)
- Use the same online tool (ResFinder) and the same version (4.4.2)
- Apply the same settings, such as identity and coverage cut-off thresholds, selected species etc., as specified in the EQA3 protocol, when submitting the reads
- Follow the instruction on how to report the obtained findings (by downloading the text files and reporting all the genes and PMs listed instead of reporting the output on the website)

4.3.1. Genes reported in *Campylobacter* samples

Table 10 includes genes that were reported in the reference dataset. All additional genes and PMs reported by the participants, but not in the reference dataset, can be found in supplementary tables (Annex C) that also contains the cases where gene variants were reported by the participants (e.g. *tetO* and *tet(O/M/O)*).

Table 10. Genes reported in Campylobacter samples EQA3-C24-01, EQA3-C24-02 and EQA3-C24-03. Reference dataset, Ref, is shaded grey. Participants are presented in numerical order. The letter "X" indicates the reported gene. Percentage concordance (%C) is based on the following scale: darkest orange colour: 100% concordance among participants, lighter orange colour: 90-99% concordance, lightest orange colour: 80-89% concordance. Concordance lower than 80% is without colour.

EQA3-C24-01																									
	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39	%C
aadE-Cc	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	96
blaOXA-489	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	96
tet(O)	X		X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	88
EQA3-C24-02																									
	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39	%C
ant(6)-Ia	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
aph(3')-III	X	X	X	X	X	X		X	X	X		X	X		X	X	X	X	X	X			X		X	78
blaOXA-193	X	X	X			X		X	X	X	X	X	X	X	X	X	X	X	X	X			X		X	78
cat(pC194)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	96
erm(B)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	100
tet(O/32/O)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X		96
EQA3-C24-03																									
	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39	%C
aph(2'')-If	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	96
aph(3')-III	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			88
blaOXA-193	X	X	X			X	X	X	X	X	X	X		X	X	X	X	X	X	X		X	X		X	79
cat	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	96
tet(O)	X	X	X		X	X	X	X	X	X			X	X	X	X	X	X	X	X	X	X	X	X	X	88

Participant E35 did not perform analysis of sample EQA3-C24-02 due to insufficient DNA quality.

Eight out of 14 genes in the three *Campylobacter* samples were reported by more than 90% of the participants.

It is clear that some participants did not follow the EQA3 protocol and applied their own criteria for reporting, which resulted in lower concordance for some genes. For example, participant E05 specified that they did not report genes *tet(O)* in sample EQA3-C24-01, *blaOXA-193* in sample EQA3-C24-02 and both *blaOXA-193* and *tet(O)* in sample EQA3-C24-03, because they had an identity of less than 100%. However, the percentage identities were all above 90% and thus should have been reported, according to the instructions in the EQA3 protocol.

Participant E28 noted that the reported genes were detected in contigs, indicating that the analysing methods was deviating from the one described in the EQA3 protocol. Participant E23 reported that a variant of *aph(3')-III* gene, *aph(3')-IIIa* was detected using AMRFinderPlus and CARD, which also indicates a deviating analytical approach.

For sample EQA3-C24-03, three participants, E05, E20 and E21 did not report the *tet(O)* gene that was detected by the EQA provider at 99.9% identity and 100% coverage. As described above, participant E05 did, in fact, detect the gene, but chose not to report it. When the quality of reads submitted by participants E20 and E21 was assessed by the EQA provider, using a local pipeline (described in section 2.5), it was observed that the N50 parameter for this sample was lower than recommended 30 for both laboratories (29 and 19, vs EQA provider: 50) (see Figure 8 and Table S8). However, when the EQA provider analysed the reads submitted by participant E20 in ResFinder (following the protocol), the *tet(O)* gene was also detected with 99.9% identity and 100% coverage. It is therefore unknown why the gene was not reported by this participant. For participant E21, the *tet(O)*

gene was not detected by the EQA provider in the reads submitted by the participant, when ResFinder was used according to the protocol. But when the EQA provider lowered the thresholds for identity and coverage it was possible to detect the *tet(O)* with an identity of 77.19% and a coverage of 77.45%. Other related genes for this sample included two hits for *tet(O/32/O)*, at identity of 87.86% and of 39.84%. This finding could suggest that a high genome fragmentation in this sample for participant E21, as indicated by the highest contig number of all participants (Figure 8), could affect whether the *tet(O)* gene was detected or not. On the other hand, participants E33 and E36 also scored low on N50 number for sample EQA3-C24-01 (Table S8) and yet, reported the *tet(O)* gene (Table 10).

4.3.2. Point mutations reported in Campylobacter samples

The point mutations reported by the participants are presented in Table 11. Only PMs in the reference dataset are included in this table, whereas additional PMs reported by the participants can be found in supplementary tables (Annex C), including cases where gene variants were reported (e.g., *gyrA* T86I and *gyrA_2* T86I).

Table 11. Point mutations (PMs) reported in Campylobacter samples. Reference dataset, Ref, is shaded grey. Participants are presented in numerical order. The letter "X" indicates the reported PM. Percentage concordance (%C) is based on the following scale: darkest orange colour: 100% concordance among participants, lighter orange colour: 90-99% concordance, lightest orange colour: 80-89% concordance. Concordance lower than 80% is without colour.

EQA3-C24-01																									
	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39	%C
23S A2075G	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X		X	91
gyrA T86I	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	96
EQA3-C24-02																									
	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39	%C
gyrA T86I	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	95
rpsL K43R	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	100
EQA3-C24-03																									
	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39	%C
gyrA T86I	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	100

Participant E35 did not perform analysis of sample EQA3-C24-02 for point mutations due to insufficient DNA quality.
Participant E37 did not perform analysis of any samples for point mutations due to insufficient DNA quality.

All five PMs present in the three samples were identified by more than 90% of participants. Based on participant's comments, participant E05 did not report the 23S A2075G substitution in sample EQA3-C24-01, because the query sequence was less than 100% identical with the reference sequence (99.48%). This percentage identity was identical for the EQA provider in the reference dataset. The EQA protocol (Section 9.1.1) specified that all point mutations listed in the summary file, downloaded from the ResFinder website after analysis, should be reported. The same mutation was not reported in the same sample by participant E31, who reported that the mutation was listed as "undefined". ResFinder does add these comments to the mutations, but only on the website. In the summary file, that participants were asked to report from, the detected mutations are simply listed without additional comments.

Participant E19 was forced to use AMRFinderPlus, as PointFinder, reportedly, did not work.

4.4. Predicted phenotypes for *Campylobacter* samples

The tables below show the predicted phenotypes established by the EQA provider and the results reported by the participants for all three *Campylobacter* samples. According to the protocol (Section 9.1.1), the participants were asked to report "the Resistant WGS-predicted phenotype for the antimicrobials included in the predicted phenotype question in the reporting scheme." The options in the reporting scheme included "wild type" (WT - sensitive), "non-wild type" (NWT – resistant) and "not determined" (ND). The latter option

could be used if ResFinder did not give a WT or NWT prediction. This was the case for Chloramphenicol phenotype prediction in all samples (Table 12 to 14), where the EQA provider reported "ND".

In the tables below, the WT and NWT phenotype predictions that diverge from the EQA provider's reported predictions, are marked with a blue colour. The ND option, which diverges from the EQA provider's prediction, is marked in yellow.

Table 12. Predicted phenotypes in sample EQA3-C24-01. WT – wild type, NWT – non wild type, ND – not determined. Blue colour: WT and NWT phenotype predictions diverging from the EQA provider's reported predictions. Yellow colour: ND diverging from the EQA provider's prediction.

	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39
Ampicillin	WT	WT	WT	WT	WT	WT	WT	WT	WT	NWT	WT	WT	WT	WT	WT	WT	WT	WT		WT	NWT	WT	WT		WT
Chloramphenicol	ND	WT	WT	ND	WT	WT	WT	ND	ND	WT	WT	ND	WT	WT	ND	WT	WT			ND	ND	WT	WT		WT
Ciprofloxacin	NWT	NWT	NWT	ND	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	ND	NWT	WT	NWT	NWT		NWT
Erythromycin	NWT	NWT	NWT	ND	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	ND	NWT	WT	NWT	NWT		NWT
Gentamicin	WT	WT	WT	ND	WT	WT	WT	WT	WT	NWT	WT	WT	WT	WT	WT	WT	WT	WT		WT	NWT	WT	WT		WT
Tetracycline	NWT	NWT	NWT	ND	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	ND	NWT	WT		NWT

Participant E37 did not detect any phenotypes

Table 13. Predicted phenotypes in sample EQA3-C24-02. WT – wild type, NWT – non wild type, ND – not determined. Blue colour: WT and NWT phenotype predictions diverging from the EQA provider's reported predictions. Yellow colour: ND diverging from the EQA provider's prediction.

	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39
Ampicillin	WT	NWT	WT	WT	NWT	WT	NWT	WT	WT	NWT	WT	WT	WT	WT	WT	WT	NWT	WT		NWT	NWT		WT		WT
Chloramphenicol	ND	NWT	WT	ND	NWT	WT	NWT	ND	ND	NWT	NWT	ND	NWT	NWT	ND	NWT	NWT	ND		ND	ND		NWT		NWT
Ciprofloxacin	NWT	NWT	NWT	ND	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	ND	NWT	WT		NWT		NWT
Erythromycin	NWT	NWT	NWT	ND	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT		NWT		NWT
Gentamicin	WT	WT	WT	ND	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT		WT	NWT		WT		WT
Tetracycline	NWT	NWT	NWT	ND	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT		WT		NWT		NWT

Participant E35 reported too low DNA quality to report results; Participant E37 did not detect any phenotypes

Table 14. Predicted phenotypes in sample EQA3-C24-03. WT – wild type, NWT – non wild type, ND – not determined. Blue colour: WT and NWT phenotype predictions diverging from the EQA provider's reported predictions. Yellow colour: ND diverging from the EQA provider's prediction.

	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39
Ampicillin	WT	NWT	WT	WT	NWT	WT	WT	WT	WT	NWT	WT	WT	NWT	WT	WT	WT	NWT	WT		WT	NWT	NWT	NWT		WT
Chloramphenicol	ND	NWT	WT	ND	NWT	WT	NWT	ND	ND	NWT	NWT	ND	NWT	NWT	ND	NWT	NWT	ND		ND	ND	WT	NWT		NWT
Ciprofloxacin	NWT	NWT	NWT	ND	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	ND	NWT	WT	NWT	NWT		NWT
Erythromycin	WT	WT	WT	ND	WT	WT	WT	WT	WT	WT	WT	WT	WT	NWT	WT	WT	WT	WT		WT	NWT	WT	WT		WT
Gentamicin	NWT	NWT	NWT	ND	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT		NWT
Tetracycline	NWT	NWT	NWT	ND	NWT	NWT	NWT	NWT	NWT	NWT	WT	WT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	NWT	WT	NWT	NWT		NWT

Participant E37 did not detect any phenotypes

For all *Campylobacter* samples, it was observed that participant E33 systematically reported results that were "reverse" to the provider's results, apart from the "ND" result for Chloramphenicol in all three samples. It could be guessed that participant E33 had swapped the abbreviations "WT" and "NWT".

Otherwise, there were only a few divergent results compared to the EQA provider. Most divergent results were observed for Chloramphenicol. In samples EQA3-C24-02 and EQA3-C24-03, the Chloramphenicol-resistance associated genes, *cat(pC194)* and *cat*, respectively, are present, and were reported by the majority of participants (Table 10). As a consequence, many participants (11/23) reported the NWT predicted phenotype for these samples. The EQA provider, however, along with 8 participants, reported "ND" (not determined) phenotype for all three samples.

5. Overall evaluation of AMR determinant detection and reporting

In order to evaluate the ability of participants to correctly detect and report *Salmonella* and *Campylobacter* genes and PMs, we calculated a "reporting percentage" of all genes and PMs for both species for each participant compared to EQA providers results. To identify possible reasons for differences between the participants' and the EQA provider results, we correlated the "reporting percentage" with participants' sequencing quality metrics, as well as with adherence to the provided protocol (Figure 12).

For this overall evaluation, we categorised the participants in three groups based on the sequencing quality results and protocol adherence. A participant received a "QC not passed" status if one of the three strains of specific species did not pass the sequencing QC thresholds. A participant received a "protocol not pass" status if they did not adhere to the EQA3 protocol. Examples of that are participants that used assemblies in addition to reads or participants using different ResFinder thresholds than those recommended in the EQA3 protocol. Lastly, participants that did not belong to the above categories, received a "pass QC/protocol" category.

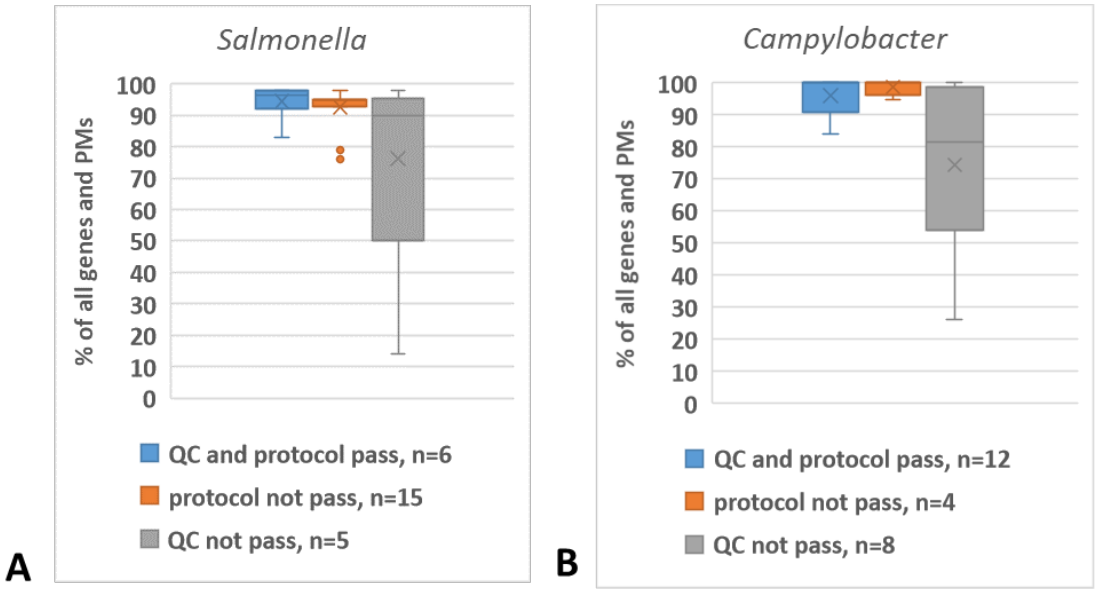

Figure 12. The distribution of total number of targets (genes and PMs) identified by all participants in three Salmonella (A) and in three Campylobacter (B) strains. The participants are divided into three categories: QC/protocol pass – sequencing was successful for all three strains; protocol not pass – protocol was not followed in at least one occasion in any strain; QC not pass – at least one of the sequencing QC parameters did not pass the threshold for at least one strain.

This comparison revealed that participants that produced good quality sequences for all *Salmonella* and *Campylobacter* samples and followed the protocol were able to identify nearly all genes and PMs correctly (*Salmonella*, M=95%, SD=6%; *Campylobacter*, M=96%, SD=6%).

Participants that performed high quality sequencing but did not follow the provided protocol, also had overall high reporting percentage for both *Salmonella* and *Campylobacter* (*Salmonella*, M=93%, SD=6%; *Campylobacter*, M=99%, SD=3%).

The lowest reporting percentage was observed for those participants that failed in the sequencing QC for at least one sample of specific species. For *Salmonella*, an average reporting percentage was 76% (SD=35%) and for *Campylobacter* it was 74% (SD=27%). High standard deviation for both *Salmonella* and *Campylobacter* indicates high variation of reporting percentage in this category. We could not investigate the reasons of variation for all participants, and therefore only a case-by-case evaluation was performed and was communicated to individual participants (data not shown).

With the above analyses we demonstrate that the sequencing quality has the highest impact on participants' ability to correctly detect and report genes and PMs in *Salmonella* and *Campylobacter*. To overcome this, improvements in sequencing procedures in some of the laboratories that participated in this EQA are needed.

6. Conclusions

The EQA3-WGS-AMR, organised by the Statens Serum Institut in collaboration with the Technical University of Denmark, is the third and final EQA exercise in the FWD AMR-RefLabCap project. The overall aim of this EQA was to compare the participant's ability to identify genes and PMs that confer AMR in *Salmonella* and *Campylobacter* using WGS, based on provided DNA samples and a harmonised analytical approach.

For guidelines concerning sequencing quality, participants were recommended to follow the standard protocol for AMR gene detection and clone identification (https://www.fwdamr-reflabcap.eu/resources/reflabcap-protocols-and-guidelines).

In this third EQA, the participants were asked to use a harmonised protocol for detection of AMR markers determination and reporting, created for the purpose of this EQA (Section 9.1.1). The reason for this was to minimize the impact of using different databases, thresholds and reporting approaches and evaluate whether the differences in reporting of genes and PMs could be attributed to the quality of sequencing performed by each participant.

Based on the analysis of the reported results, it was shown that the sequencing quality had an effect on how well the participants were able to identify AMR genes and PMs (see section 5 for details).

The kit used for DNA extraction had an effect on one QC parameter (number of contigs). Participants who used the Illumina DNA Prep library preparation kit had less variable number of contigs at 25X coverage (Figure 4 and Figure 9) compared to other kits such as the Illumina Nextera XT Library Prep kit.

Even though many participants have their preferred and implemented bioinformatic pipelines for detection of AMR markers, in this EQA the participants were asked to use the online ResFinder tool (http://genepi.food.dtu.dk/resfinder) due to its accessibility and ease of use. The EQA3 protocol specified that one type of file (fastq) should be used as input to ResFinder, the selected version of software should be (4.4.2), the default thresholds for identity and coverage should be 90% and 60% respectively, and, importantly, the participants should report their results in a unified way.

Based on the reported gene and PM results as well as the participants' comments, we could conclude that not all participants adhered to the provided EQA3 protocol. Some participants still reported using other tools, such as AMRFinderPlus or CARD, other file input types (fasta instead of fastq) or applying thresholds different from the ones specified in the EQA3 protocol. This resulted in some genes not getting reported (sections 3.3.1 and 4.3.1) or reporting more genes than in the reference data set (Annex C). The predicted phenotype reporting was an optional part in this EQA, but many participants used this option. In this part, there were only a few results diverging from the reference dataset, mostly related to not following the protocol.

To sum up, the participants were generally able to sequence the provided DNA samples with a high quality and report the correct AMR genes and point mutations. The percentage of correct results varied depending on the sequencing quality. Not all participants followed the EQA3 protocol, which in this EQA, did not result in large variations. However, when participating in an EQA exercise, it would be beneficial for the EQA provider if the protocol was followed , so that any introduced changes, compared to previous rounds, could be evaluated properly.

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8. Annex A

8.1. Supplementary material on QC metrics for both organisms

Table S 1. DNA measurement instruments, kits and concentrations reported by 26 participants and EQA provider (REF) for Salmonella samples.

Participant code	DNA measurment		DNA concentration, ng/µl		
	Instruments	Kits	EQA3-S24-01	EQA3-S24-02	EQA3-S24-03
REF[A]	Qubit	1X dsDNA HS	50.5	25.0	13.4
E02[B]	Qubit	dsDNA BR	31.0	31.1	23.3
E03	Quant-iT/Clariostar	dsDNA HS/-	23.1	22.7	18.8
E05	Quantus Fluorometer	-	23.0	42.0	27.0
E06[C]	Qubit	dsDNA BR	68.5	115.2	75.5
E10	Qubit	1X dsDNA HS	40.8	37.4	24.3
E11	Qubit	dsDNA BR	38.5	63.1	13.1
E14	Qubit	dsDNA HS	37.0	39.4	29.4
E15[D]	Qubit	dsDNA BR	38.6	27.4	20.5
E16	Qubit	dsDNA BR	3.3	33.4	22.9
E17	Qubit	dsDNA BR	39.0	32.3	21.5
E19	Quantus Fluorometer	QuantiFluor® ONE dsDNA System	21.0	227.0	32.0
E20[A]	Qubit	1x dsDNA HS	35.0	36.0	26.0
E21[E]	Qubit	1x ds DNA HS	15.0	24.0	22.4
E22[B]	Qubit	dsDNA HS	40.2	30.4	25.2
E23	Qubit	dsDNA BR	3.0	34.8	23.5
E24[A]	Qubit	dsDNA BR	273.0	68.2	48.2
E28	Qubit	Quant-iT PicoGreen dsDNA	81.3	92.6	88.3
E29[A]	Qubit	-	33.3	39.2	24.5
E31[A]	Qubit	dsDNA BR	20.0	36.0	25.2
E33	Qubit	Qubit	10.4	7.3	7.0
E35	Qubit	1X dsDNA HS	15.3	10.4	8.1
E36[A]	Qubit	dsDNA HS	29.7	40.8	27.5
E37	Qubit/Quant-iT/Nanodrop	Thermo Qubit kit/BiODynamyKit/-	281.0	236.0	24.3
E38[A]	Qubit	dsDNA HS	21.1	41.7	29.8
E39	Qubit	dsDNA HS	41.5	40.9	57.0
E40[B]	Qubit	dsDNA HS	34.2	36.0	22.8

[A]Indicates that participant used 2 µL DNA and 198 µL Working Solution for the concentration measurement
[B]Indicates that participant used 1 µL DNA and 199 µL Working Solution for the concentration measurement
[C]Indicates that participant used 20 µl DNA after 2x dilution of 2 µl pure DNA for the concentration measurement
[D]Indicates that participant used 5 µl DNA and 195 µL Working Solution for the concentration measurement
[E]Participant indicated a concentration used for WGS of 10 ng/µl

Table S 2. DNA measurement instruments, kits and concentrations reported by 24 participants and EQA provider (REF) for Campylobacter samples.

Participant code	DNA measurment		DNA concentration, ng/µl		
	Instruments	Kits	EQA3-C24-01	EQA3-C24-02	EQA3-C24-03
Ref[A]	Qubit	1x dsDNA HS	69.3	131.2	56.3
E02[B]	Qubit	dsDNA BR	51.1	47.5	16.3
E03	Quant-iT/Clariostar	dsDNA HS/-	29.5	27.6	23.5
E05	Quantus Fluorometer	-	70.0	59.0	17.0
E09[AC]	Qubit	1x dsDNA HS	85.2	56.6	37.5
E10	Qubit	1x dsDNA HS	24.6	83.2	44.0
E12	-	-	-	-	-
E14	Qubit	dsDNA HS	75.6	72.4	56.0
E16	Qubit	dsDNA BR	63.2	158.0	24.6
E19	Quantus Fluorometer	QuantiFluor One dsDNA System	80.0	74.0	68.0
E20	Qubit	1x dsDNA HS	26.0	31.0	7.0
E21	Qubit	1x dsDNA HS	75.0	65.8	6.4
E22[B]	Qubit	dsDNA HS	77.8	78.2	112.0
E23	Qubit	dsDNA BR	41.9	15.2	3.0
E24[A]	Qubit	dsDNA BR	112.0	115.0	33.9
E27[A]	Qubit/Nanodrop	dsDNA BR/-	60.4	78.9	35.7
E28	Qubit	Quant-iT PicoGreen dsDNA	72.6	41.3	46.3
E29[AD]	Qubit	-	3.4	6.1	37.3
E31[A]	Qubit	dsDNA BR	78.8	82.8	15.8
E32	Qubit	1x dsDNA HS	51.0	53.9	4.4
E33	Qubit	Qubit	2.3	20.9	16.5
E35	Qubit	1x dsDNA HS	11.9	0.0	7.2
E36[A]	Qubit	dsDNA HS	47.9	132.0	32.3
E37	Qubit/Quant-iT/Nanodrop	Qubit/BiODynamyKit/	193.0	210.0	139.0
E39	Qubit	dsDNA HS	55.0	54.0	35.5

[A]Indicates that participant used 2 µL DNA and 198 µL Working Solution for the concentration measurement
[B]Indicates that participant used 1 µL DNA and 199 µL Working Solution for the concentration measurement
[C]Participant indicated that samle 01 was diluted 1:2, and sample 02-1:10 before the measurement
[D]Participant indicated that samples 01 and 02 were diluted 10x before the measurement

Table S 3. Distribution of sequencing quality parameters among 26 participants and EQA provider for sample EQA3-S24-01. Indicative QC ranges show QC parameters recommended by FWD AMR-RefLabCap agreed WGS protocol (in yellow), and recommended by internal QC pipeline at SSI (not highlighted) for Salmonella. The participant and the values that did not pass QC thresholds according to agreed WGS protocol are highlighted in red. The values that did not pass additional QC thresholds are indicated in red font.

QC parameters	% Species 1	% Species 2	% uncla-ssified	Length at >25X (in Mbp)	Length at <25X (in Kbp)	Contigs at 25X	Contigs at <25X	Average coverage	Reads (in thousands)	Average read length	Average insert size	N50 (in Kbp)
Indicative QC ranges		<5%		4.4-5.8	<250	>0	<1000	>30				>30
REF	93.7	0.8	4.8	5.1	6.0	127	8	127	4655	142	271	97
E02	94.7	0.9	3.7	5.1	0.0	46	0	125	4462	148	400	418
E03	95.2	0.7	3.5	5.1	0.0	50	0	107	3783	146	296	381
E05	89.8	1.0	7.9	0.2	4559.8	58	2962	14	482	168	152	2
E06	97.5	0.4	1.6	5.1	1.3	68	1	89	3032	149	363	231
E10	97.3	1.0	1.1	5.1	0.0	42	0	111	2081	274	337	640
E11	94.8	1.0	3.3	5.1	0.0	42	0	59	1297	240	429	710
E14	96.8	0.9	1.7	5.1	0.0	70	0	79	1845	221	228	402
E15	93.1	0.8	5.5	5.1	0.0	84	0	94	3540	138	232	180
E16	96.5	0.7	2.2	5.1	0.0	42	0	66	2284	149	395	533
E17	95.3	0.9	3.1	5.1	0.0	44	0	127	2426	276	351	640
E19	89.7	0.7	9.1	5.1	0.0	64	0	190	8082	122	202	412
E20	92.7	1.0	5.7	5.1	7.9	185	10	86	3034	148	278	61
E21	94.7	0.7	3.9	5.0	21.2	274	33	89	3149	146	275	36
E22[A]	94.4	3.2	1.4	5.1	-	175	53	101	1698	308	-	178
E23	96.0	0.8	2.6	5.1	0.0	61	0	110	3800	149	291	383
E24[A]	92.4	4.5	2.4	5.1	-	94	5	60	1000	309	-	214
E28	96.0	0.8	2.6	5.1	0.0	52	0	145	5078	147	346	383
E29	95.6	1.0	2.7	5.1	0.0	60	0	103	2547	212	282	267
E31	96.5	0.8	2.2	5.1	0.0	55	0	153	5342	147	291	533
E33	91.7	1.0	6.8	0.3	4703.3	42	354	18	706	132	248	23
E35	97.0	0.3	2.4	3.6	1535.9	45	49	29	1003	149	523	116
E36	95.8	0.8	2.7	5.0	33.9	199	32	61	1318	241	423	46
E37[B]	0.89	0.2	79.82	NA	-	NA	NA	NA	1	203	-	NA
E38	96.7	1.1	1.3	5.1	0.0	40	0	248	4522	283	414	640
E39	94.7	0.8	3.8	5.1	0.0	56	0	186	6621	146	306	533
E40	90.7	0.7	8.0	5.1	0.0	175	0	319	16966	97	262	61

[A]indicated participants used IonTorrent technology
[B]indicated participant used Nanopore technology

Table S 4. Distribution of sequencing quality parameters among 26 participants and EQA provider for sample EQA3-S24-02. Indicative QC ranges show QC parameters recommended by FWD AMR-RefLabCap agreed WGS protocol (in yellow), and recommended by internal QC pipeline at SSI (not highlighted) for Salmonella. The participant and the values that did not pass QC thresholds according to agreed WGS protocol are highlighted in red. The values that did not pass additional QC thresholds are indicated in red font.

QC parameters	% Species 1	% Species 2	% uncla-ssified	Length at >25X (in Mbp)	Length at <25X (in Kbp)	Contigs at 25X	Contigs at <25X	Average coverage	Reads (in thousands)	Average read length	Average insert size	N50 (in Kbp)
Indicative QC ranges		<5%		4.4-5.8	<250	>0	<1000	>30				>30
REF	93.7	0.8	4.8	5.1	6.0	127	8	127	4655	142	271	97
E02	94.7	0.9	3.7	5.1	0.0	46	0	125	4462	148	400	418
E03	95.2	0.7	3.5	5.1	0.0	50	0	107	3783	146	296	381
E05	89.8	1.0	7.9	0.2	4559.8	58	2962	14	482	168	152	2
E06	97.5	0.4	1.6	5.1	1.3	68	1	89	3032	149	363	231
E10	97.3	1.0	1.1	5.1	0.0	42	0	111	2081	274	337	640
E11	94.8	1.0	3.3	5.1	0.0	42	0	59	1297	240	429	710
E14	96.8	0.9	1.7	5.1	0.0	70	0	79	1845	221	228	402
E15	93.1	0.8	5.5	5.1	0.0	84	0	94	3540	138	232	180
E16	96.5	0.7	2.2	5.1	0.0	42	0	66	2284	149	395	533
E17	95.3	0.9	3.1	5.1	0.0	44	0	127	2426	276	351	640
E19	89.7	0.7	9.1	5.1	0.0	64	0	190	8082	122	202	412
E20	92.7	1.0	5.7	5.1	7.9	185	10	86	3034	148	278	61
E21	94.7	0.7	3.9	5.0	21.2	274	33	89	3149	146	275	36
E22[A]	94.4	3.2	1.4	5.1	-	175	53	101	1698	308	-	178
E23	96.0	0.8	2.6	5.1	0.0	61	0	110	3800	149	291	383
E24[A]	92.4	4.5	2.4	5.1	-	94	5	60	1000	309	-	214
E28	96.0	0.8	2.6	5.1	0.0	52	0	145	5078	147	346	383
E29	95.6	1.0	2.7	5.1	0.0	60	0	103	2547	212	282	267
E31	96.5	0.8	2.2	5.1	0.0	55	0	153	5342	147	291	533
E33	91.7	1.0	6.8	0.3	4703.3	42	354	18	706	132	248	23
E35	97.0	0.3	2.4	3.6	1535.9	45	49	29	1003	149	523	116
E36	95.8	0.8	2.7	5.0	33.9	199	32	61	1318	241	423	46
E37[B]	0.89	0.2	79.82	NA	-	NA	NA	NA	1	203	-	NA
E38	96.7	1.1	1.3	5.1	0.0	40	0	248	4522	283	414	640
E39	94.7	0.8	3.8	5.1	0.0	56	0	186	6621	146	306	533
E40	90.7	0.7	8.0	5.1	0.0	175	0	319	16966	97	262	61

[A]indicated participants used IonTorrent technology
[B]indicated participant used Nanopore technology

Table S 5. Distribution of sequencing quality parameters among 26 participants and EQA provider for sample EQA3-S24-03. Indicative QC ranges show QC parameters recommended by FWD AMR-RefLabCap agreed WGS protocol (in yellow), and recommended by internal QC pipeline at SSI (not highlighted) for Salmonella. The participant and the values that did not pass QC thresholds according to agreed WGS protocol are highlighted in red. The values that did not pass additional QC thresholds are indicated in red font.

QC parameters	% Species 1	% Species 2	% uncla-ssified	Length at >25X (in Mbp)	Length at <25X (in Kbp)	Contigs at 25X	Contigs at <25X	Average coverage	Reads (in thousands)	Average read length	Average insert size	N50 (in Kbp)
Indicative QC ranges		<5%		4.4-5.8	<250	>0	<1000	>30				>30
REF	93.5	1.9	4.5	5.3	5.0	183	8	118	4548	142	269	75
E02	94.8	2.0	3.0	5.3	0.0	88	0	99	3682	148	388	273
E03	93.1	1.8	5.0	5.3	0.0	89	0	107	4191	139	262	272
E05	89.4	2.0	7.8	0.0	4973.7	46	3016	14	464	172	159	3
E06	97.0	1.8	1.1	0.0	5166.4	24	706	17	610	150	450	12
E10	96.7	2.3	0.8	5.3	0.0	73	0	71	1369	281	392	280
E11	94.5	2.6	2.6	5.3	0.7	75	1	62	1401	240	424	280
E14	96.5	1.9	1.5	5.3	0.0	130	0	66	1689	210	214	223
E15	92.7	1.8	5.2	5.3	0.7	140	1	118	4769	135	218	92
E16	96.7	1.6	1.6	5.3	0.0	89	0	110	3964	149	360	239
E17	95.9	2.1	1.8	5.3	0.0	84	0	120	2392	273	333	278
E19	92.1	1.5	6.4	5.3	0.0	102	0	211	9006	127	219	271
E20	92.7	1.7	5.4	5.3	0.0	135	0	89	3297	148	291	104
E21	94.5	1.6	3.8	5.3	19.5	288	27	90	3369	144	266	36
E22[A]	95.9	2.9	0.9	5.3	-	190	2	93	1609	310	-	223
E23	95.9	1.4	2.6	5.3	0.0	94	0	72	2617	149	321	239
E24[A]	95.1	3.2	1.5	5.3	-	175	1	58	999	312	-	224
E28	96.0	1.7	2.2	5.3	0.0	90	0	130	4745	148	364	239
E29	94.9	2.1	2.8	5.3	3.3	113	4	99	2465	223	315	122
E31	96.6	1.6	1.6	5.3	0.0	94	0	167	6064	148	294	239
E33	91.2	1.4	7.4	5.0	261.8	253	118	33	1387	127	216	33
E35	97.1	1.0	1.8	3.8	1487.3	71	60	30	1092	149	465	120
E36	93.3	2.2	4.0	5.3	30.6	254	28	61	1306	261	313	44
E37[B]	95.7	2.0	1.9	5.5	-	8	0	212	462	2521	-	5013
E38	96.2	2.1	1.3	5.3	0.0	69	0	233	4452	285	464	280
E39	95.1	1.8	2.9	5.3	0.0	89	0	161	5975	146	310	278
E40	90.9	1.5	7.5	5.3	0.0	291	0	314	17444	97	256	37

[A]indicated participants used IonTorrent technology
[B]indicated participant used Nanopore technology

Table S 6. Distribution of sequencing quality parameters among 24 participants and EQA provider for sample EQA3-C24-01. Indicative QC ranges show QC parameters recommended by FWD AMR-RefLabCap agreed WGS protocol (in yellow), and recommended by internal QC pipeline at SSI (not highlighted) for Campylobacter. The participant and the values that did not pass QC thresholds according to agreed WGS protocol are highlighted in red. The values that did not pass additional QC thresholds are indicated in red font.

QC parameters	% Species 1	% Species 2	% uncla-ssified	Length at >25X (in Mbp)	Length at <25X (in Kbp)	Contigs at 25X	Contigs at <25X	Average coverage	Reads (in thousands)	Average read length	Average insert size	N50 (in Kbp)
Indicative QC ranges		<5%		1.5-1.9	<250	>0	<1000	>30				>30
REF	91.9	1.7	6.3	1.7	0.0	75	0	309	4159	129	181	106
E02	94.8	2.2	2.8	1.7	0.0	46	0	129	1541	148	398	162
E03	93.7	2.4	3.9	1.7	0.0	55	0	109	1360	139	264	140
E05	87.7	3.1	7.5	1.7	0.8	173	3	34	406	161	150	23
E09	95.9	2.6	1.4	1.7	0.0	49	0	2928	18916	269	364	162
E10	96.4	2.8	0.7	1.7	0.0	45	0	126	781	278	358	173
E12	90.2	2.6	4.9	1.7	0.0	70	0	945	12714	149	202	140
E14	96.4	2.1	1.4	1.7	0.0	75	0	217	1782	211	214	140
E16	96.3	2.0	1.6	1.7	0.0	52	0	355	4115	149	336	162
E19	86.4	1.9	11.6	1.7	0.0	64	0	1117	16303	121	204	140
E20	93.1	1.8	4.9	1.7	0.0	60	0	131	1593	144	243	140
E21	88.3	1.6	10.0	1.7	6.6	307	18	152	2234	117	140	12
E22[A]	96.3	2.4	1.3	1.7	-	155	1	219	1231	308	-	106
E23	95.2	2.3	2.4	1.7	0.0	51	0	88	1026	149	319	140
E24	88.5	1.4	9.9	1.7	0.0	241	0	1550	26469	101	105	18
E27	95.7	2.3	1.6	1.7	0.0	48	0	116	731	277	355	140
E28	95.7	2.1	2.2	1.7	0.0	39	0	135	1594	148	408	173
E29	95.7	2.4	1.7	1.7	0.0	50	0	241	1886	223	326	140
E31	95.9	2.2	1.8	1.7	0.0	57	0	577	6760	148	295	140
E32	95.0	2.2	2.6	1.7	0.0	43	0	465	3226	250	337	140
E33	90.4	2.8	6.7	1.6	92.8	79	19	29	397	128	207	52
E35	95.7	2.5	1.8	1.7	0.0	34	0	41	485	149	600	173
E36	93.5	2.7	2.9	1.7	37.2	156	36	46	331	245	273	21
E37[B]	89.7	7.6	1.4	1.8	-	3	0	173	141	2275	-	1698
E39	95.5	2.1	2.3	1.7	0.0	56	0	503	6020	145	266	140

[A]indicated participant used IonTorrent technology
[B]indicated participant used Nanopore technology

Table S 7. Distribution of sequencing quality parameters among 24 participants and EQA provider for sample EQA3-C24-02. Indicative QC ranges show QC parameters recommended by FWD AMR-RefLabCap agreed WGS protocol (in yellow), and recommended by internal QC pipeline at SSI (not highlighted) for Campylobacter. The participant and the values that did not pass QC thresholds according to agreed WGS protocol are highlighted in red. The values that did not pass additional QC thresholds are indicated in red font.

QC parameters	% Species 1	% Species 2	% uncla-ssified	Length at >25X (in Mbp)	Length at <25X (in Kbp)	Contigs at 25X	Contigs at <25X	Average coverage	Reads (in thousands)	Average read length	Average insert size	N50 (in Kbp)
Indicative QC ranges		<5%		1.5-1.9	<250	>0	<1000	>30				>30
REF	88.8	5.4	5.1	1.8	0.0	74	0	244	3185	138	233	142
E02	88.8	6.9	3.6	1.8	0.0	53	0	96	1198	148	392	148
E03	87.0	7.0	5.5	1.8	0.0	67	0	127	1687	136	253	142
E05	84.8	6.7	7.1	1.8	0.0	111	0	49	602	159	150	82
E09	89.1	8.2	1.9	1.8	0.0	55	0	2782	18747	269	363	142
E10	90.1	8.0	1.1	1.8	0.0	48	0	110	710	281	385	148
E12	85.0	6.2	5.0	1.8	0.0	77	0	761	10473	148	205	142
E14	90.0	7.1	2.2	1.8	0.0	85	0	117	1049	202	203	107
E16	90.0	6.9	2.4	1.8	0.0	55	0	347	4201	149	346	118
E19	80.9	5.6	12.9	1.8	0.0	75	0	950	15207	114	170	142
E20	85.6	4.7	9.1	1.7	42.2	72	24	54	713	138	308	81
E21	85.8	5.0	8.6	1.7	32.8	313	61	162	2282	126	166	10
E22[A]	89.6	7.9	1.8	1.8	-	117	0	237	1366	312	-	107
E23	89.2	6.9	3.2	1.8	0.0	59	0	91	1102	149	300	142
E24	88.3	5.8	5.3	1.8	0.0	86	0	679	9615	127	166	107
E27	89.3	8.2	1.7	1.8	0.0	57	0	194	1299	271	327	142
E28	89.1	7.1	3.1	1.8	0.0	53	0	103	1262	148	405	156
E29	87.9	6.1	5.3	1.8	0.0	57	0	327	3134	191	250	136
E31	89.9	6.9	2.6	1.8	0.0	66	0	512	6259	148	291	142
E32	89.6	6.8	2.8	1.8	0.0	40	0	274	1720	293	594	170
E33	86.6	5.2	7.6	1.4	373.8	194	189	30	420	127	204	9
E35												
E36	86.1	5.9	7.1	1.8	0.4	74	1	153	1437	195	234	86
E37[B]	90.1	2	6.7	1.8	-	3	0	74	69	1899	-	1192
E39	91.0	5.5	2.9	1.8	0.0	55	0	757	9264	148	310	142

[A]indicated participant used IonTorrent technology
[B]indicated participant used Nanopore technology

Note: The percentage of reads of sample EQA3-C24-02 mapped with kraken to a different species (% Species 2) was for many participants higher than the recommended 5%. However, the second species for this *C. coli* sample was *C. jejuni* and therefore, was not treated as contamination.

Table S 8. Distribution of sequencing quality parameters among 24 participants and EQA provider for sample EQA3-C24-03. Indicative QC ranges show QC parameters recommended by FWD AMR-RefLabCap agreed WGS protocol (in yellow), and recommended by internal QC pipeline at SSI (not highlighted) for Campylobacter. The participant and the values that did not pass QC thresholds according to agreed WGS protocol are highlighted in red. The values that did not pass additional QC thresholds are indicated in red font.

QC parameters	% Species 1	% Species 2	% uncla-ssified	Length at >25X (in Mbp)	Length at <25X (in Kbp)	Contigs at 25X	Contigs at <25X	Average coverage	Reads (in thousands)	Average read length	Average insert size	N50 (in Kbp)
Indicative QC ranges		<5%		1.5-1.9	<250	>0	<1000	>30				>30
REF	94.8	0.5	4.5	1.8	0.0	49	0	238	3029	139	240	98
E02	95.8	0.5	3.4	1.8	0.0	34	0	133	1611	148	432	114
E03	95.6	0.5	3.7	1.8	0.0	44	0	94	1171	143	284	127
E05	92.2	0.6	6.6	1.7	2.7	121	5	42	508	158	148	47
E09	97.6	0.6	1.6	1.8	0.0	43	0	3212	21108	269	357	167
E10	98.0	0.6	1.1	1.8	0.0	37	0	110	692	281	392	167
E12	92.4	2.2	4.5	1.8	0.0	63	0	1223	16349	149	193	114
E14	97.1	0.6	2.1	1.8	0.0	60	0	179	1611	197	198	127
E16	97.2	0.5	2.1	1.8	0.0	43	0	320	3791	149	351	127
E19	83.4	0.4	16.1	1.8	0.0	53	0	604	9601	113	204	114
E20	93.8	0.5	5.5	1.7	7.7	125	17	140	1751	142	223	29
E21	91.7	0.4	7.6	1.7	15.2	174	23	176	2428	128	180	19
E22[A]	97.6	0.6	1.5	1.8	-	94	1	221	1192	328	-	106
E23	96.4	0.5	2.9	1.8	0.0	49	0	79	953	147	298	128
E24	93.1	0.5	6.3	1.8	0.0	65	0	845	12147	123	159	114
E27	97.6	0.7	1.5	1.8	0.0	41	0	169	1104	271	334	153
E28	97.0	0.5	2.3	1.8	0.0	43	0	150	1801	148	369	127
E29	91.0	0.5	8.3	1.8	0.2	60	1	387	4373	157	179	128
E31	97.3	0.5	2.1	1.8	0.0	49	0	539	6441	148	291	114
E32	97.1	0.5	2.2	1.8	1.9	26	1	362	2281	282	512	212
E33	91.6	0.5	7.8	1.7	62.3	154	50	45	630	125	191	20
E35	97.7	0.6	1.6	1.8	0.0	37	0	76	902	150	531	114
E36	96.3	0.5	2.9	1.7	49.2	132	42	115	921	217	294	26
E37[B]	85.8	0.7	11.9	0	-	0	31	11	22	1128	-	73
E39	94.6	0.5	4.8	1.8	0.0	57	0	824	10920	133	211	113

[A]indicated participant used IonTorrent technology
[B]indicated participant used Nanopore technology

9. Annex B

9.1. Supplementary materials, methods for gene and point mutation detection

9.1.1. EQA3 protocol

This document contains the protocol to follow when reporting genes and point mutations from your sequenced samples. Even though you may have used ResFinder before, please make sure to read and follow this protocol.

The final page (page 5) contains the protocol for upload of read files to our ftp server.

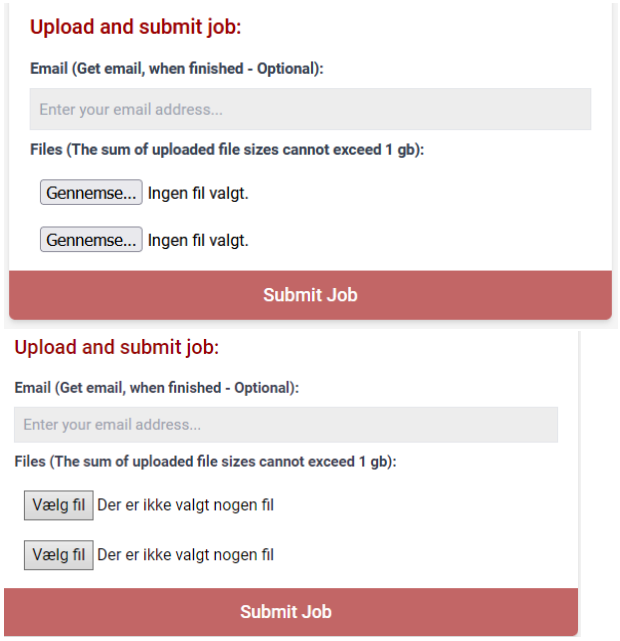
1. Open the website http://genepi.food.dtu.dk/resfinder
2. **Select ResFinder version 4.4.2**, even though there is a newer version available on the website.
3. Leave the default thresholds for chromosomal point mutations and acquired antimicrobial resistance genes (90% ID and 60% minimum length)
4. Do not select any additional options for chromosomal point mutations
5. Do not select the Disinfectant option
6. In the box "Species and input data type", select the relevant species:

Salmonella	***Campylobacter*** **(please select the species that you identified)**
Species and input data type: Select species Salmonella spp.*	Species and input data type: Select species Campylobacter jejuni* Species and input data type: Select species Campylobacter coli*

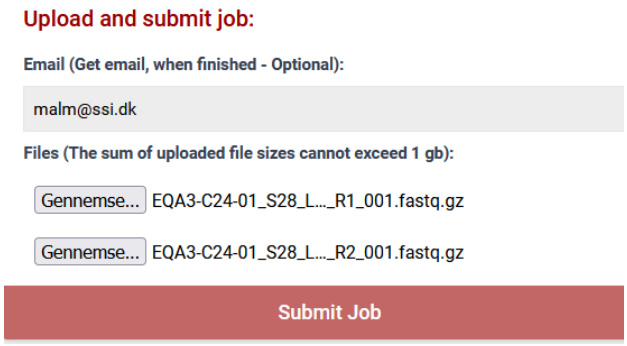
7. Select the relevant FASTQ data type:

Non Nanopore reads (f. ex. Illumina)	**Nanopore reads**
Select input type FASTQ (Non-nanopore Reads)	Select input type FASTQ (Nanopore Reads)

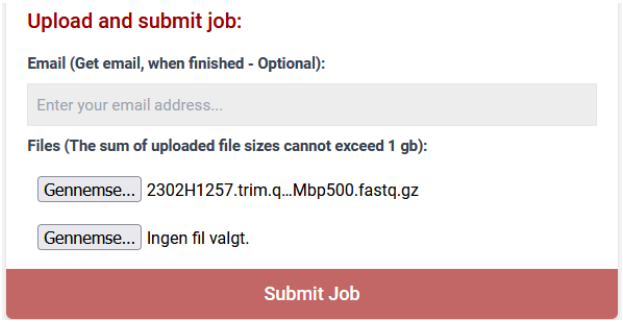
8. Enter your email address and select the read files you want to upload (one file at a time) by pressing the button "Gennemse…" or "Vælg fil" in this window (depending on the browser, the text in the buttons might be different):



For paired-end Illumina reads, one pair of read files (R1 and R2) can be uploaded at a time (example below):



For Nanopore reads, a read file representing one isolate should be uploaded at a time (example):

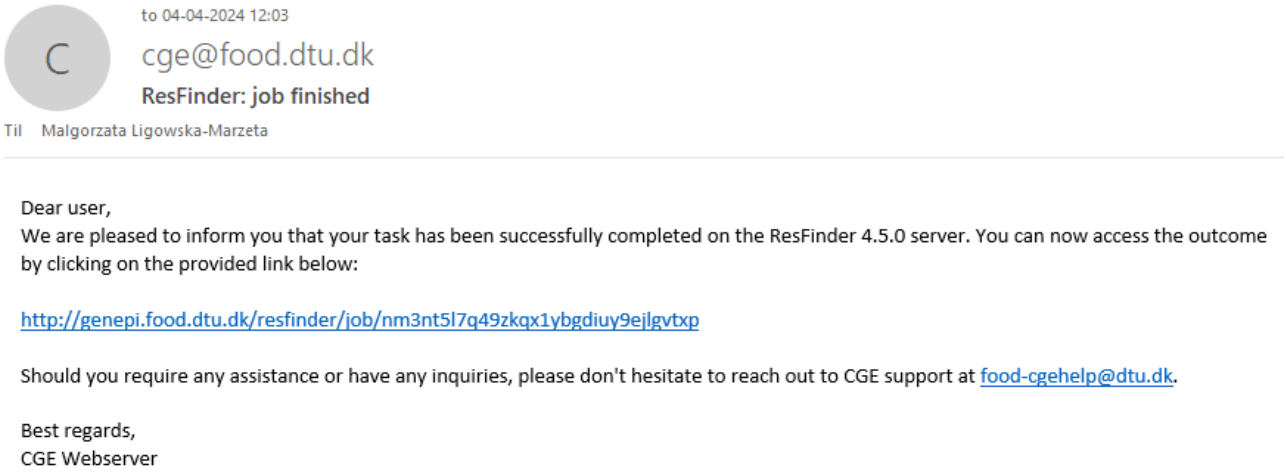


9. Press Submit Job and wait. After a while you will see the following:

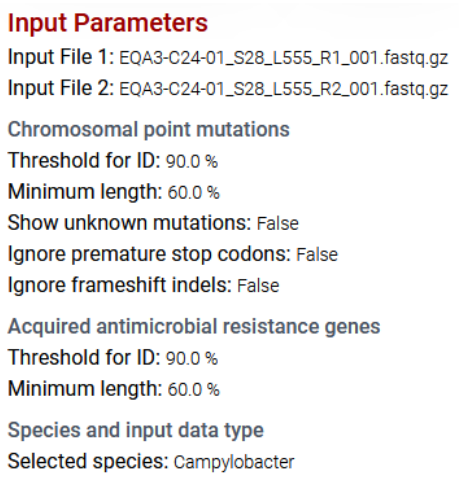
If you stay on this website, the results will eventually appear. Simultaneously, you will receive an email with a link to your results (see point 10).

If you now press on the DTU logo in the upper left corner, you will go back to the main ResFinder website and be able to submit the next sequences.

10. When the job on ResFinder is finished, you will receive the following email (example):

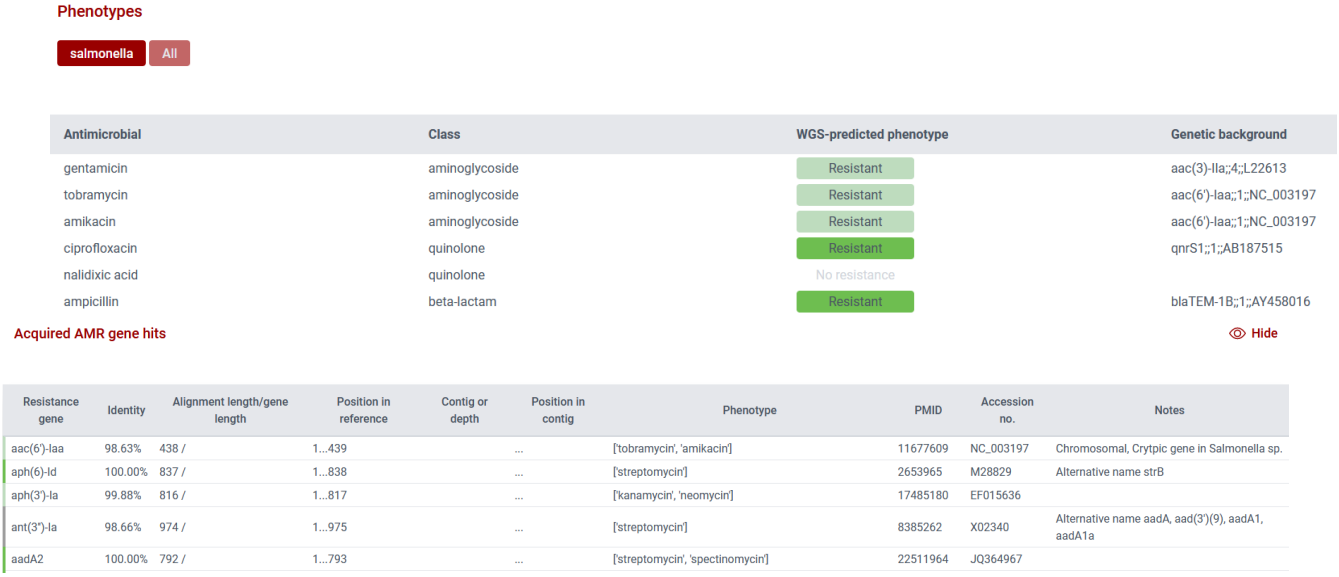


11. Upon clicking on the link, you will open a website with your results. In order to check which isolate the results are for, **scroll to the bottom of the website**, where you can see the Input Parameters and the name of your sample (example):



12. The results page is divided into the following parts:
    a. Phenotypes
    b. Acquired AMR gene hits
    c. Chromosomal mutations mediating AMR
    d. Acquired disinfectant resistance hits
    e. Downloads
    f. Input parameters

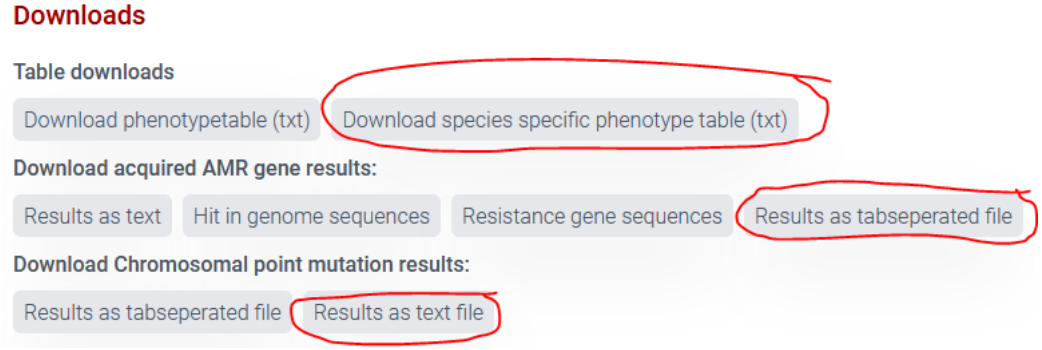
13. Here's an example of an output (please only look at species-relevant site: "salmonella" or "campylobacter" and not "All"):



The colours mean the following:

Green: 100% identity over 100% of the length
Light green: <100% identity over 100% of the length
Grey: <100% identity over <100% of the length

14. Scroll down to the "Downloads" section and download and save the following three files, circled in red, for all samples:



15. You are now ready to report your results in the reporting scheme:

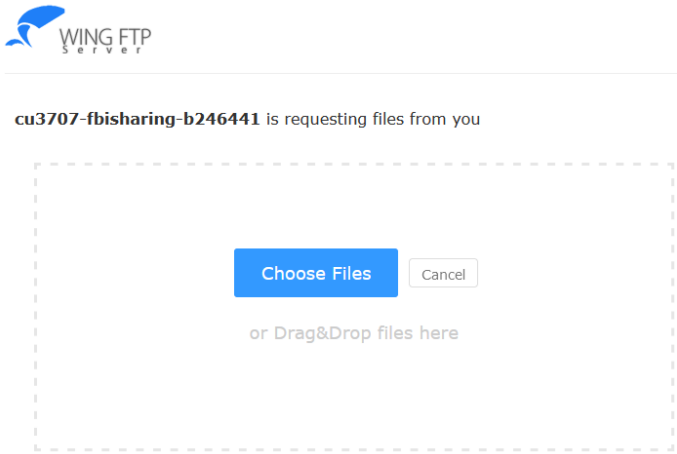
    a. From the file with acquired AMR gene results, report all the genes in that file.
    b. From the file with Chromosomal point mutations, report all the point mutations in that file.
    c. From the phenotype table (txt) file – report the Resistant WGS-predicted phenotype **for the antimicrobials included in the predicted phenotype question in the reporting scheme**.

Upload of reads to an ftp server for participant

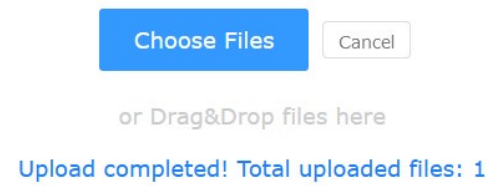
You are asked to upload the reads that you obtained in your laboratory, so that the EQA provider can perform QC analysis on them. Please name the reads so that it is clear which DNA sample they represent. Each participant has a separate folder for upload that the other participants cannot access.

1. Click on the personal link below:
   The personal link for upload for each laboratory can be found in the protocol sent to you in an email on the 10th of April.

2. You will see the following:



3. Drag and Drop your reads or click on “Choose Files” and select your read files. Please do not compress your reads (for example, by zipping them).
4. When you see this message in the window, your upload is complete and you can close the website:



Thank you!

9.2. Supplementary materials, serotype/species and ST identification

Table S 9. Salmonella serotypes and methods used for serotype identification by the participants

Lab code	SeqSero	SISTR	ST/eBG (EnteroBase)	Other	EQA3-S24-01	EQA3-S24-02	EQA3-S24-03	Comment
E02	X	X			Rissen	Heidelberg	Typhimurium monophasic	
E03	X				Rissen	Heidelberg	I 4,[5],12:i:-	
E05	X				Rissen	Heidelberg	Monophasic Typhimurium	
E06				X	Rissen	Heidelberg	Monophasic variant of S. Typhimurium (4,5,12:i:-)	
E10	X				Rissen	Heidelberg	Monophasic Typhimurium	
E11	X	X			Rissen (6,7,14:f,g:-)	Heidelberg (1,4,[5],12:r:1,2)	Monophasic Typhimurium (I 4,[5],12:i:-)	
E14	X	X			Rissen	Heidelberg	Typhimurium monophasic	
E15	X				Rissen	Heidelberg	Monophasic Typhimurium	
E16				X	Rissen	Heidelberg	monophasic Typhimurium	
E17	X	X			Rissen	Heidelberg	1,4,[5],12:i:-	
E19	X				Rissen	Heidelberg	Nonophasic variant of Typhimurium	
E20	X				Rissen	Heidelberg	potential monophasic variant of Typhimurium	
E21	X		X		Rissen	Heidelberg	4,5,12:i:-	
E22	X	X			Rissen	Heidelberg	I 1,4, [5],12:i:- (monophasic variant of Typhimurium)	
E23	X				Rissen	Heidelberg	monophasic variant of Typhimurium	
E24	X			X	S. Rissen	S. Heidelberg	monophasic variant of S. Typhimurium	
E28	X				Rissen	Heidelberg	I 1,4,[5],12:i:- / Typhimurium	
E29	X				Rissen	Heidelberg	I 4,[5],12:i:- monophasic S. typhimurium	
E31	X				Rissen (7:f,g:-)	Heidelberg(4:r:1,2)	potential monophasic variant of Typhimurium(4:i:-)	
E33	X				Rissen	Heidelberg	I 4,[5],12:i:-	
E35	X				Rissen 7:f,g:-	Heidelberg 4:r:1,2	I 4,[5],12:i:-	
E36	X				Rissen	Heidelberg	I 1,4,[5],12:i:-	
E37	X				Paratyphi A	NA	NA	
E38	X	X			Rissen	Heidelberg	4,[5],12:i:- (Previously described as monophasic S.Typhimurium	
E39	X				Antigenic profile - 7:f,g:- ; O-7, H1-f,g, H2-	Antigenic profile - 4:r:1,2 ; O-4, H1-r, H2-1,2	Antigenic profile - 4:i:- ; O-4, H1-i, H2-	
E40	X				Rissen	Heidelberg	I -:i:-	flagellar factor correct, but no reporting of O antigen

NA – serotype not reported

Table S 10. Salmonella ST and methods used for identification by the participants

Lab code	MLST method	EQA3-S24-01	EQA3-S24-02	EQA3-S24-03
E02	MLST (tsemann)	469	15	34
E03	MLST (tsemann)	469	15	34
E05	Enterobase, MLST2.0 (CGE tools)	469	15	34
E06	Enterobase	469	15	34
E10	MLST (tsemann)	469	15	34
E11	Enterobase	469	15	34
E14	MLST2.0 (CGE tools)	469	15	34
E15	SeqSphere	469	15	34
E16	MLST2.0 (CGE tools)	469	15	34
E17	SeqSphere	469	15	34
E19	SeqSphere	469	15	34
E20	MLST2.0 (CGE tools)	469	15	34
E21	MLST (tsemann)	469	15	34
E22	MLST2.0 (CGE tools), SeqSphere	469	15	34
E23	MLST2.0 (CGE tools)	469	15	34
E24	Enterobase	469	15	34
E28	MLST (tsemann)	469	15	34
E29	MLST (tsemann)	469	15	34
E31	MLST (tsemann)	469	15	34
E33	MLST (tsemann)	469		34
E35	MLST2.0 (CGE tools)	469	15	34
E36	MLST2.0 (CGE tools), SeqSphere	469	15	34
E37	MLST (tsemann)			
E38	BioNumerics, SeqSphere, Other	469	15	34
E39	MLST2.0 (CGE tools)	469	15	34
E40	MLST (tsemann)	469	15	34

10. Annex C

10.1. Supplementary gene tables for both organisms

This section contains tables with genes that were reported by some participants but not by the EQA provider.

Table S 11. EQA3-S24-01 additional genes (not reported in the reference dataset)

	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40
aac(3)-IId		X			X		X					X				X		X			X		X	X	X	X	
aac(6')-Ib3																								X			
mdsA*,mdsB* (efflux)							X																				
blaOXA-232																											X

Table S 12. EQA3-S24-02 additional genes (not reported in the reference dataset)

	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40
tetM		X			X		X									X		X									
qacL		X																									
mdsA							X																				
mdsB							X																				

Table S 13. EQA3-S24-03 additional genes (not reported in the reference dataset)

	Ref	E02	E03	E05	E06	E10	E11	E14	E15	E16	E17	E19	E20	E21	E22	E23	E24	E28	E29	E31	E33	E35	E36	E37	E38	E39	E40
aadA2b																		X									
aac(6')_Ib_cr					X																						
mcr9					X		X																				
aac(3)-IIg							X									X											
blaTEM 232 - 96.4%																									X		
mdsA/B (efflux)							X																				
arr							X																				

Table S 14. EQA3-C24-01 additional genes (not reported in the reference dataset)

	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39
aph(2'')-If																								X	
blaOXA-61																								X	
tet(O/M/O)		X																							

Table S 15. EQA3-C24-02 additional genes (not reported in the reference dataset)

	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39
ant(9)-Ia														X											
aph(3')-IIIa							X																		
blaOXA-61					X		X													X				X	
tet(O)																									X
blaOXA-450			X		X																				
sat --> AMRFinder + CARD														X											
blaOXA-451									X																

Table S 16. EQA3-C24-03 additional genes (not reported in the reference dataset)

	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39
aadE-Cc																								X	
aph(3')-IIIa							X							X											X
blaOXA-61					X								X									X			
blaOXA-450					X				X																

10.2. Supplementary point mutation tables for both organisms

This section contains tables with genes that were reported by some participants but not by the EQA provider. No additional point mutations were reported for *Salmonella*.

Table S 17. EQA3-C24-01 additional point mtuations (not reported in the reference dataset)

	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39
gyrA_2 p.T86I		X																							

Table S 18. EQA3-C24-02 additional point mtuations (not reported in the reference dataset)

	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39
gyrA_2 p.T86I		X																							

Table S 19. EQA3-C24-03 additional point mtuations (not reported in the reference dataset)

	Ref	E02	E03	E05	E09	E10	E12	E14	E16	E19	E20	E21	E22	E23	E24	E27	E28	E29	E31	E32	E33	E35	E36	E37	E39
50S_L22 A103V														X											

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