EU Reference Laboratory for Antimicrobial Resistance
External Quality Assurance System (EQAS) 2024

PROTOCOL

for confirmation and characterisation of methicillin-resistant *Staphylococcus aureus* (MRSA) by PCR or other genotypic testing methods

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1 INTRODUCTION

EFSA has announced the launch of an EU-wide baseline survey protocol for a coordinated monitoring program on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in pigs in 2025. One of the goals of this baseline survey is to determine the prevalence of MRSA in fattening pigs in Europe and across EU member states. The MRSA Baseline Survey is described in detail in the document **Technical specifications for a baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in pigs** (EFSA, 2022). For this baseline survey, member states will be required to isolate MRSA from pig nasal swabs, and to confirm and characterise these presumptive MRSA samples either by PCR or WGS methodologies. The samples are to be analysed according to the laboratory protocols available on the European Union Reference Laboratory on Antimicrobial resistance (EURL-AR) website (www.eurl-ar.eu), which includes a protocol for isolation of MRSA from animals or environment, two multiplex PCR's for the initial characterisation of the collected isolates and a protocol for traditional *spa*-typing. To ensure the quality of results reported in the baseline survey, the EURL-AR will launch this proficiency test (PT) for the MRSA confirmation and characterisation by PCR, which can also be completed using WGS. The PT is aimed only at the genomic characterisation of MRSA and there will be no assessment of MRSA isolation procedures.

2 OBJECTIVES

This proficiency test aims to assess the laboratory setup for confirmation and characterisation of presumptive MRSA isolates, including assessment of positive controls selected for the multiplex PCR methods. The participating laboratories can use this PT to ensure that their setup is prepared for the MRSA baseline survey in 2025. Further objectives are to evaluate and improve the comparability of surveillance data on MRSA reported to EFSA by different laboratories.

3 OUTLINE OF THE MRSA PCR PT 2024

3.1 Shipping, receipt and storage of samples

In March 2024, the EURL-AR sent out an online questionnaire to the National Reference Laboratories for Antimicrobial Resistance (NRL-AR), to get information on their intensions to collect results for the MRSA Baseline by PCR or WGS, as well as their interest in participating in the present PT and/or in receiving the PCR control strains selected by the EURL-AR.

In late April 2024, the NRLs will receive a parcel containing 3 PCR control strains and/or 10 test strains for the PT from the DTU, National Food Institute. All strains belong to UN3373, Biological substance, category B. Participants should expect that MRSA strains will be included in the samples.

All strains will be shipped as swabs of pure cultures in transport media. Upon arrival to your laboratory, store the strains in a dark place at 5°C to 25°C until further microbiological analysis. To simulate the flow of the isolation protocol, the strains should be sub-cultured on blood agar and tested

for typical morphology, as well as purity, before being subjected to PCR. Please note that some control- and test- strains might not grow on selective agar for MRSA.

3.2 Overview of control strains

Three control strains (Table 1) are selected for the MRSA multiplex PCR-1 and -2 (protocols available at https://www.eurl-ar.eu/protocols.aspx). All three control strains are *Staphylococcus aureus* and are positive for the *spa* gene. All three control strains have previously been sent out as part as the EURL-AR AST EQAS, and have the EQAS number in their strain ID.

Table 1: List of control strains selected for the MRSA multiplex PCRs

Control strain ID	Positive control for	Species	MRSA
PCR-1-C1 EURL ST-12.7	PVL, *scn*, *spa*	*S. aureus*	No
PCR-1-C2 EURL ST-11.3	*mecA*, CC398, *spa*	*S. aureus*	Yes
PCR-2-C3 EURL ST 17.7	*mecC*, *spa*	*S. aureus*	Yes

Two strains are selected as controls for the PCR-1, covering the five genes of this multiplex PCR setup. The PCR-1-C1 strain is not an MRSA (and will not grow on chromogenic screening plate for MRSA), whereas the PCR-1-C2 is an MRSA by presence of *mecA.* One additional strain is selected as positive control for the PCR-2 and is an MRSA and positive control for *mecC.*

In the previous version of the multiplex PCR-2, the original EURL-AR protocol for PCR detection of *mecA* and *mecC,* other control strains were suggested (*mecA* positive *S. aureus* 50A247, *mecC* positive *S. aureus* LGA251 and *S. aureus* ATCC 29213 as control for *spa*). These strains can still be used as positive controls e.g. if they are already part of the PCR setup of the NRL. For the purpose of this PT, please use the three control strains listed in Table 1.

3.3 Overview of test strains

Ten *Staphylococcus spp.* strains are included in this PT setup as test strains and some of these are defined as MRSA by the presence of either of the genes *mecA* or *mecC.*

Table 2: Codes for the test strains included in the current PT

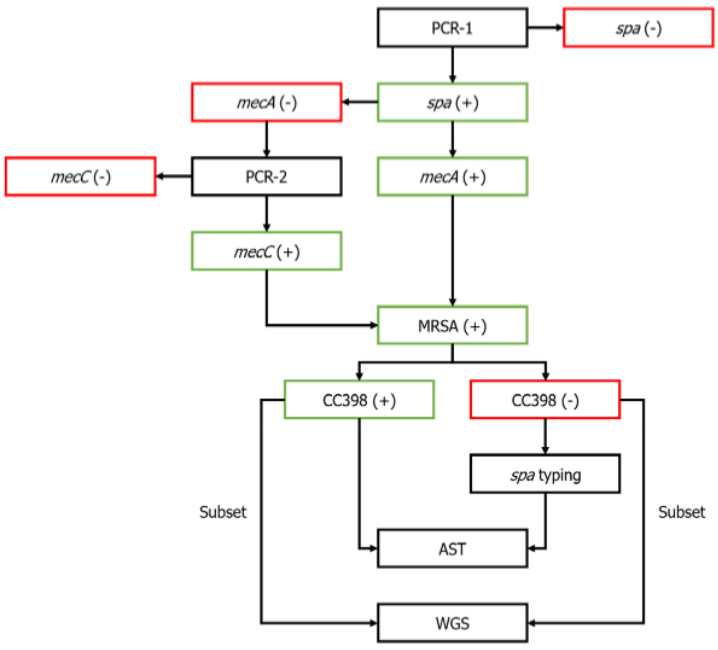
Test strains
EURL MRSA-PT-01
EURL MRSA-PT-02
EURL MRSA-PT-03
EURL MRSA-PT-04
EURL MRSA-PT-05
EURL MRSA-PT-06
EURL MRSA-PT-07
EURL MRSA-PT-08
EURL MRSA-PT-09
EURL MRSA-PT-10

3.4 Confirmation and characterisation of MRSA by PCR assays

Participants in this PT are expected to perform the confirmation and characterisation of the test strains, following the multiplex PCR assay setup (Figure 1) described in the EFSA Technical specifications in section 6.3 (EFSA, 2022). The PCR protocols are available on the EURL-AR website (https://www.eurl-ar.eu/protocols.aspx). The protocols provide examples for DNA extraction methods, PCR reagents and gels, but the NRL's are not limited to following these suggestions, and can use their normal PCR setup for the PT.

Participants who prefer to test their WGS setup rather than using PCR are welcome to perform the WGS analysis of the test strains (see section 6.5 in EFSA, 2022) and report their results based on this. This PT does only cover the genes relevant to the multiplex PCR assays. The suggested PCR assays are developed as a fast screening for the most expected characteristics of the presumptive MRSAs collected during the MRSA baseline survey, to limit the number of strains that will need to be further characterised by *spa*-typing and/or WGS. The PCR assays will essentially allow to confirm species, MRSA genotypes, and a CC398-specific gene region for which a positive PCR result will render further *spa*-typing irrelevant.

The flow of the PCR assays can be seen in Figure 1 (Copied from Figure 3 in EFSA, 2022). The first PCR; PCR-1 is used to confirm the species as *S. aureus* by the *spa* gene, and further to detect the presence of *mecA* and other relevant genetic factors for characterisation (CC398, PVL and *scn*; see EFSA, 2022 Section 6.3 for details). Isolates that are positive for the *spa* gene but negative for the *mecA* gene are subsequently subjected to PCR-2 in order to determine the presence or absence of the *mecC* gene.



AST: antimicrobial susceptibility testing; MRSA: methicillin-resistant *Staphylococcus aureus*; WGS: whole genome sequencing.

Figure 1 Flowchart for confirmatory testing, typing, antimicrobial susceptibility testing and WGS of MRSA; from Figure 3 in EFSA, 2022.

3.5 Confirmation and characterisation of MRSA by WGS methodologies

The participating NRLs can also test their WGS analysis setup, in preparation for the MRSA Baseline survey, using the supplied test strains and can report their findings in the same way as if they had performed the PCR assays.

The Technical specifications (EFSA, 2022) describes how the WGS analysis will allow a broader characterisation of the MRSA strains' genetic determinants involved in AMR, host adaptation and virulence. Specifically, there is a list of mandatory genotypic characteristics in relation to multi-locus sequence typing (MLST), staphylococcus cassette chromosome *mec* (SCCmec) typing, detection of AMR genes and point mutations and detection of other genes associated with host adaptation and virulence (see section 6.6 in EFSA, 2022) primarily using webtools available at cge.food.dtu.dk. These tools can be incorporated into internal pipelines and other tools and pipelines can also be used to obtain the same genetic information. The laboratories are suggested to use the SPAdes tool for assembly, version 3.14 or newer version, according to the WGS protocol, and this webtool is available here: https://cge.food.dtu.dk/services/SPAdes-3.14/.

The EURL-AR has developed a 'database' (based on the gene list from Table 9 in EFSA, 2022) which is consisting of a compiled list of representative fasta files of the relevant genes (selected in collaboration with Statens Serum Institut, SSI, Denmark), including also the relevant genes from the PCR assays (*mecA*, *mecC*, the CC398 specific gene region, *scn* and PVL; see Appendix 2) which can be used as a template for gene detection using in-house pipelines or analyses. The *spa* gene variants are not included in the database, and the *spa* type must be confirmed by other tools.

This database can also be used with the 'MyDBFinder' tool on the CGE website (https://cge.food.dtu.dk/services/MyDbFinder/) and will allow for a quick genomic screening of the genes relevant for this PT.

The database is supplied as a text-file in fasta format and will also be made available on the EURL-AR website (See Appendix 2).

Guidance for use of MyDBFinder:

Save the database as a fasta or txt-file (MyDBFinder_MRSA_Baseline.fasta) on your local computer. When accessing the webtool, this file should be selected as user database as the first step:

1) **Upload user database**

   Select the database file (.txt or .fasta format) as user database

2) **Select relevant settings for the analysis**

Referring to the Tech. spec. (EFSA, 2022) Table 9, use the settings:

- **Threshold for % ID: 90%**
- **Minimum length in %: 60%**

3) Select the type of sequence file

Select either assembled genome or reads, depending on the input type.

4) Upload your sequence

Select sequences from your folders. Please note that you can only upload sequences from one isolate at a time, either one fasta file or two paired-end read files.

5) Output from MyDBFinder

The output will be in the format known from ResFinder, with information about the fasta header (gene name and description), % Identity, Query/template length, contig no., and position in contig. The threshold of acceptance is 90 % ID and 60 % minimal length (coverage), as defined by the settings, and as such any match should be reported.

Thus, for the CC398-specific gene region, the EURL-AR recommends accepting only 100 % gene identity (Figure 2). If the output identity is < 100 %, the clonal complex (CC) type should be confirmed by other tools, e.g. by MLST.

Database				
Fasta header	**Identity**	**Query / Template length**	**Contig**	**Position in contig**
czrC| cadmium_and_zinc_resistance_gene_C| AB505629.1| Staphylococcus_aureus_strain_JCSC6944	99.95	1935 / 1935	NODE_17_length_17108_cov_23.8624_ID_2346	3557..5491
mecA_4|beta-lactam-inducible_penicillin-binding_protein_A|CP053070.1| Staphylococcus_aureus_strain_HL20709	99.95	2007 / 2007	NODE_18_length_16254_cov_30.0195_ID_2348	11287..13293
sau1-hsdS1_CC398| restriction_endonuclease_subunit_S| JF781577.1| Staphylococcus_aureus_strain_CC398	100	487 / 487	NODE_3_length_391424_cov_29.401_ID_2318	340263..340749

Figure 2: Example of output from MyDBFinder tool, using the 'MyDBFinder_MRSA_Baseline' database, showing positive results for *mecA* and CC398 in relation to this PT and additionally a positive result for *czrC*.

Only the genes relevant for the multiplex PCR assays will be reported in this PT (See Appendices 1 and 2).

4 REPORTING OF RESULTS AND EVALUATION

Test forms are available for recording your results before entering them into the data collection survey (See Appendix 1 and additional file provided by the EURL-AR).

The EURL-AR will launch an online questionnaire for results reporting on May 2, 2024. By this date, the data questionnaire will be available following this link:

https://ec.europa.eu/eusurvey/runner/EURLAR_MRSA_PT_2024

Results must be submitted no later than June 20, 2024, at 16:00. If you experience difficulties in entering your results, please contact us directly.

There will be no immediate feedback from the survey tool. The expected PT results will be distributed by email to the network for self-evaluation. All results will be summarized in a report which will be publicly available. The data in the report will be presented with laboratory codes. A laboratory code is known to the individual laboratory, whereas the complete list of laboratories and their codes is confidential and known only to the EURL-AR and the EU Commission. All conclusions will be public.

If you have questions, please do not hesitate to contact the PT Coordinator:

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5 REFERENCES:

EFSA, 2022: Technical specifications for a baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in pigs. EFSA Journal 2022;20(10):7620 https://doi.org/10.2903/j.efsa.2022.7620o

Additional websites:

https://www.eurl-ar.eu/protocols.aspx

https://cge.food.dtu.dk/

https://cge.food.dtu.dk/services/MyDbFinder/

https://cge.food.dtu.dk/services/SPAdes-3.14/

6 APPENDIX 1 – TEST FORM

Example of test form for collecting results from the 3 control strains as well as the 10 test strains in the PCR PT.

Strain ID: _____

Q1: Did you obtain pure growth on blood agar with a morphology expected for a *Staphylococcus spp.* strain?

☐Yes
☐No
Comment:

_____

Q2: Select the genes which were found positive in the PCR-1:

Gene name	PCR-1
spa	☐
mecA	☐
PVL	☐
scn	☐
CC398	☐

Q3: Did you proceed to PCR-2 (isolate positive for *spa* gene but negative for *mecA*)?

☐Yes
☐No
☐Not relevant – performed WGS

Q4: Select the genes which were found positive in the PCR-2:

Gene name	PCR-2
spa	☐
mecA	☐
PVL	☐
mecC	☐

Comment:

_____

7 APPENDIX 2 – GENES IN 'MYDBFINDER_MRSA_BASELINE' DATABASE

List of genes included in the 'MyDBFinder_MRSA_Baseline' database file – see also 'Mandatory genotypic characteristics' in Table 9 in EFSA, 2022.

Gene Name	Gene Description	Reference Accession
czrC	Cadmium and zinc resistance gene C	Y00688.1
chp	Chemotaxis inhibitory protein of S. aureus (CHIPS)	DQ530361.1
***lukS*-PV**	Prophage-encoded Panton-Valentine leukocidin (PVL)	AB006796.1
***lukF*-PV**	Prophage-encoded Panton-Valentine leukocidin (PVL)	AB006796.1
scn	Staphylococcal complement inhibitor (SCIN)	DQ530361.1
sak	Staphylokinase (SAK)	DQ530361.1
sea	Staphylococcal enterotoxin A (SEA)	DQ530361.1
seb	Staphylococcal enterotoxin B (SEB)	AF410775.1
sec	Staphylococcal enterotoxin C (SEC)	X05815.1 AF217235.1 CP001996.1
sep	Staphylococcal enterotoxin P (SEP)	BA000018.3
tarP	Prophage-encoded wall teichoic acid glycosyltransferase (TarP)	BA000018.3
tsst	Staphylococcal pathogenicity island (SaPI)-encoded toxic shock syndrome toxin 1 (TSST-1)	U93688.2 AF217235.1 CP001996.1
vwbSaPI	Staphylococcal pathogenicity island (SaPI)-encoded von Willebrand factor-binding protein (genetic marker of ruminant adaptation	HM211303.1 CP001996.1 HM228920.1
IEC1	Prophage-borne immune evasion cluster encoding SCIN (genetic marker of IEC1, encoded by scn) and different combinations of CHIPS, SAK, SEA, and SEP (genetic marker of human adaptation)	This cluster consists of scn, chp, sak, sea and sep, which are all in the database
IEC1 type	A schematic representation of the different IEC1 types is provided by van Wamel et al. (2006)	This cluster consists of scn, chp, sak, sea and sep, which are all in the database