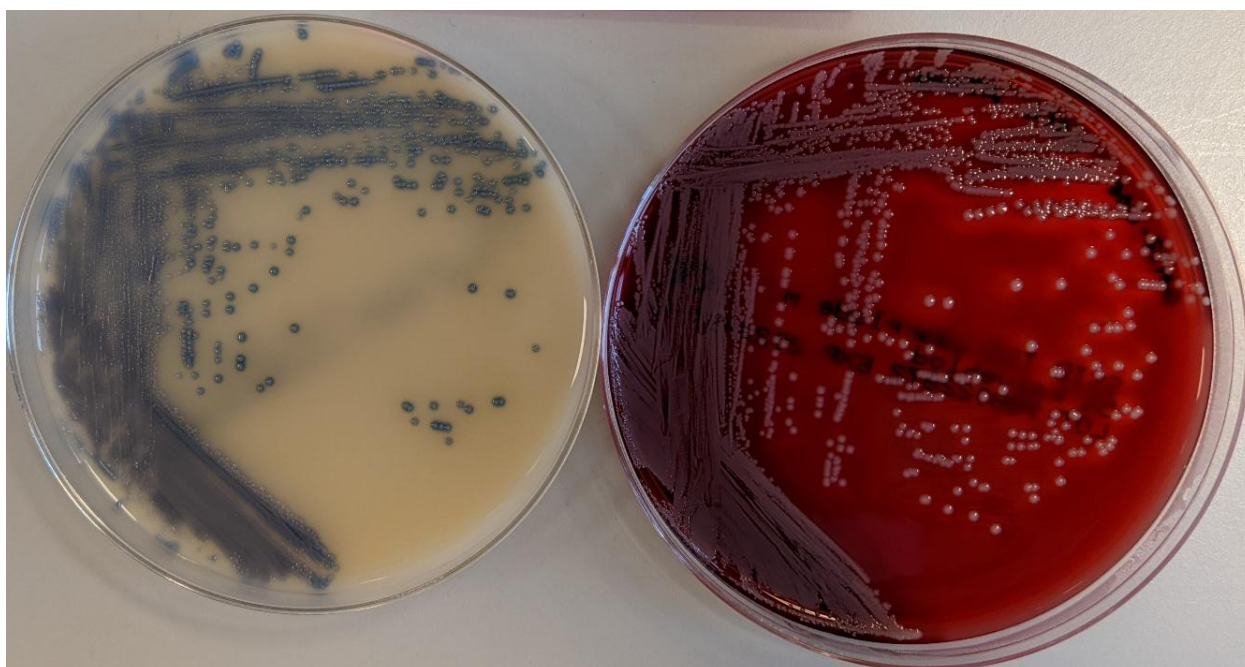




## The EURL-AR Proficiency Test for confirmation and characterisation of methicillin-resistant *Staphylococcus aureus* (MRSA) by PCR or other genotypic testing methods, 2024



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## Table of contents

<b>1</b>	<b>INTRODUCTION .....</b>	<b>3</b>
<b>2</b>	<b>OBJECTIVES .....</b>	<b>3</b>
<b>3</b>	<b>OUTLINE OF THE MRSA PCR PT 2024 .....</b>	<b>3</b>
<b>3.1</b>	<b>Shipping, receipt and storage of samples.....</b>	<b>3</b>
<b>3.2</b>	<b>Overview of control strains .....</b>	<b>4</b>
<b>3.3</b>	<b>Overview of test strains .....</b>	<b>4</b>
<b>3.4</b>	<b>Confirmation and characterisation of MRSA by PCR assays .....</b>	<b>4</b>
<b>3.5</b>	<b>Confirmation and characterisation of MRSA by WGS methodologies.....</b>	<b>5</b>
<b>4</b>	<b>PARTICIPATION AND RESULTS .....</b>	<b>5</b>
<b>4.1</b>	<b>Signup for participation and survey of use of WGS and/or PCR.....</b>	<b>5</b>
<b>4.2</b>	<b>Participation and reporting of results.....</b>	<b>5</b>
<b>4.3</b>	<b>Results .....</b>	<b>6</b>
<b>4.4</b>	<b>Concluding remarks .....</b>	<b>7</b>
<b>5</b>	<b>REFERENCES .....</b>	<b>7</b>
<b>6</b>	<b>APPENDIX 1 – SIGNUP SURVEY AND QUESTIONNAIRE PAGE 1/2 .....</b>	<b>8</b>
<b>7</b>	<b>APPENDIX 2 TEST FORM.....</b>	<b>10</b>
<b>8</b>	<b>APPENDIX 3 EXPECTED RESULTS.....</b>	<b>11</b>

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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, providing data for risk assessment and guide future monitoring and control strategies. 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](http://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 the results reported in the baseline survey, the EURL-AR launched this proficiency test (PT) for the MRSA confirmation and genomic characterisation.

## 2 OBJECTIVES

This proficiency test aimed 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; Appendix 1), to get information on their intentions to collect results for the MRSA Baseline survey 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 received a parcel containing three PCR control strains and/or ten test strains for the PT from the DTU, National Food Institute. All strains belong to UN3373, Biological substance, category B. Participants could expect that both MRSA and other *Staphylococcus* strains could be included in the samples.

### 3.2 Overview of control strains

Three control strains (Table 1) were 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 previous 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, <i>scn</i> , <i>spa</i>	<i>S. aureus</i>	No
PCR-1-C2 EURL ST-11.3	<i>mecA</i> , CC398, <i>spa</i>	<i>S. aureus</i>	Yes
PCR-2-C3 EURL ST 17.7	<i>mecC</i> , <i>spa</i>	<i>S. aureus</i>	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*. The strains can also be used as control strains for WGS and bioinformatic setups.

### 3.3 Overview of test strains

Ten *Staphylococcus spp.* strains were included in this PT as test strains and six of these were defined as MRSA by the presence of either of the genes *mecA* (four strains) or *mecC* (two strains). One additional strain carried *mecA*, but was a *Staphylococcus epidermidis* (MRSE).

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

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

### 3.4 Confirmation and characterisation of MRSA by PCR assays

Participants in this PT were expected to perform the confirmation and characterisation of the test strains, following the multiplex PCR assay setup 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 were not limited to following these suggestions, and could use their normal PCR setup for the PT.

Participants who preferred to test their WGS setup rather than using PCR could perform WGS analysis of test strains (see section 6.5 in EFSA, 2022) and report their results based on this. This PT covers the genes relevant to the multiplex PCR assays only. The suggested PCR assays are developed as a fast screening for the most expected characteristics of the presumptive MRSA's 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 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 to determine the presence or absence of the *mecC* gene.

### 3.5 Confirmation and characterisation of MRSA by WGS methodologies

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

The laboratories were suggested to use the SPAdes tool for assembly, version 3.14 or newer version, according to the WGS protocol, and could report only the PCR relevant genes in relation to this PT.

## 4 PARTICIPATION AND RESULTS

### 4.1 Signup for participation and survey of use of WGS and/or PCR

The EURL-AR launched a survey in March 2024 to ask the NRLs of their interest to participate in the MRSA PCR PT, and to ask, on behalf of EFSA, about the intentions of the NRLs to conduct the EFSA MRSA Baseline Survey for MRSA identification by WGS or PCR. Originally, 29 laboratories answered the survey, and hereof 28 intended to participate in the MRSA PT. The one laboratory which did not intend to participate, is handling food and feed samples only and has another NRL handling animal samples.

Regarding the methodology expected to be applied for MRSA identification in the Baseline Survey in 2025, nine laboratories (31%) reported to use PCR only, thirteen laboratories (45%) reported PCR and WGS, while seven laboratories (24%) reported WGS only.

### 4.2 Participation and reporting of results

A total of 27 laboratories from 26 countries reported their results in the online data collection survey within the deadline and were included in this report. From the 28 laboratories who expressed their interest in participating in the PT through the survey, two did eventually not participate, whereas one additional NRL participated in the MRSA PT. Test forms were available for recording results before entering them into the data collection survey (by EUSurvey; Appendix 2). The collection survey was opened for results reporting on May 2, 2024, and the deadline of reporting was June 20, 2024, to allow time for participants to report results after outsourced sequencing.

Twelve laboratories (44%) reported results based on PCR alone. Fifteen laboratories (56%) used WGS either alone (13 laboratories – 48%), in combination with PCR results (1 laboratory – 4%) or sequenced a subset of the test isolates only (1 laboratory – 4%). Considering the six gene included in the PT, there were 2,106 possible tests theoretically to be conducted for presence/absence of the genes

MRSA PCR PT 2024

in both the controls and the test strains. A part (3.3 %) of these results was not reported for various reasons, including one laboratory which did not receive the primers for gene *scn* and region CC398 in time for the PT exercise (26 samples excluded), and did not report findings of these genes, one laboratory only sequenced the test strains, and did not report genes for the control strains (18 samples excluded), while two laboratories did not report data on the presence or absence of the *spa* gene (26 samples excluded).

### 4.3 Results

The PT setup included testing of three positive control strains, to be used as PCR or WGS reference strains during the MRSA Baseline Survey, and ten test strains, for which the participants should report the presence or absence of the targeted genes. As such, 13 strains were screened for six genes (*PVL*, *spa*, *scn*, CC398, *mecA*, *mecC*) by 27 laboratories, with few exceptions (see 4.2). The overview of correct and incorrect reported results, according to the EURL-AR determined expected results (Appendix 3), as well as not reported results, can be seen in Table 3. The overview does not consider if the genes positive in PCR-1 were also positive in PCR-2, when this was applied.

**Table 3:** Overview of reported results of the MRSA PCR PT from 27 participants

	Tested genes						Total
	<i>spa</i>	<i>mecA</i>	<i>mecC</i>	<i>pvl</i>	<i>scn</i>	CC398	
Correctly reported	319	346	347	348	334	332	2026
Incorrectly reported	3	2	1	0	1	3	10
Not reported	29	3	3	3	16	16	70
Total	351	351	351	351	351	351	2106

Overall, ten results were incorrectly reported, accounting for 0.47 % of the results, and these deviations were related to five of the six genes tested (excluding *pvl*), with 1-3 deviations occurring for the individual genes. As such, no systematic errors appear to occur in the PCR setup. Of these ten deviations, 7 were based on PCR analysis and 3 were based on WGS (Table 4). Furthermore, 70 results (3.32 %) were not reported for various reasons (See 4.2).

**Table 4:** Overview deviations in reporting of the MRSA PCR PT

Lab number (NRL-AR-0XX)	Strain	Deviation	Method of testing
#56	PCR-1 C2	1 false negative <i>mecA</i> (in PCR-1 only)	PCR
#33	MRSA-PT-04	1 false positive <i>scn</i>	WGS
#33	MRSA-PT-04	1 false positive <i>spa</i>	WGS
#19	MRSA-PT-04	1 false positive <i>spa</i>	PCR
#64	MRSA-PT-06	1 false positive CC398	PCR
#40	MRSA-PT-08	1 false negative CC398	PCR
#40	MRSA-PT-08	1 false positive <i>mecC</i>	PCR
#25	MRSA-PT-08	1 false negative <i>spa</i>	WGS
#29	MRSA-PT-08	1 false positive <i>mecA</i>	PCR
#29	MRSA-PT-09	1 false positive CC398	PCR

The ten deviations were spread between the tested strains; one deviation occurred in a control strain (PCR-1-C2), whereas the rest were distributed between four test strains MRSA-PT-04 (n=3), MRSA-PT-06 (n=1), MRSA-PT-08 (n=4) and MRSA-PT-09 (n=1).

One control strain (PCR-1-C1) and three of the test strains, MRSA-PT-01, -06 and -08 were designated MSSA, as they were not harbouring the *mecA* or *mecC* genes, and did not have extended phenotypic resistance to beta-lactam antibiotics. Furthermore, one test strain (MRSA-PT-04) was an *S. epidermidis* carrying *mecA* (MRSE) and was thereby negative for the *S. aureus* specific *spa* gene. Two laboratories reported false positive *spa* for this strain (Table 4).

Overall, the 2,106 MRSA PCR PT results covered 873 true positive gene amplifications, and 1,269 results were true negative, which led to a PCR (or WGS) assay sensitivity of 99.64 % and a specificity of 99.45 %.

#### 4.4 Concluding remarks

With an overall result of <0.5 % deviations in the tests performed by 27 laboratories, we find the MRSA PCR/WGS gene detection setup very robust and well-implemented in the NRLs.

As some of the participating laboratories used both PCR and WGS partially or in combination to analyse the test strains, the reported results do not allow to distinguish completely between the methodology applied. Laboratories, who have reported deviating results, should carefully evaluate their laboratory results and if necessary, repeat the analysis, to elucidate if the deviation occurred by laboratory errors (e.g. mix of strains) or by errors in reporting, when entering the results in the test form or into the online data collection survey.

## 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.2022.7620>

## **6 APPENDIX 1 – SIGNUP SURVEY AND QUESTIONNAIRE PAGE 1/2**

### **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. One of the goals of this baseline survey is to determine the prevalence of MRSA in fattening pigs in the EU and across EU member states. For this, member states will be required to isolate MRSA from pig nasal swabs, and to confirm and characterise these either by PCR or WGS methodologies. To ensure the quality of reported results, the EURL-AR will launch in Spring 2024 a proficiency test for the MRSA identification and characterisation by PCR, which can also be completed using WGS. Live bacterial cultures (Approx. 10 test strains plus control strains) will be sent to participating laboratories for PCR (or WGS) characterisation. The participating laboratories will then report the findings to the EURL-AR using an online survey and the results will be evaluated.

The purpose of this questionnaire is to explore the intentions of laboratories in the EURL-AR network to deploy PCR for the MRSA identification and also the level of interest in participating in the above-mentioned proficiency test for MRSA identification by PCR (or WGS), organised by the EURL-AR.

### **CONTACT INFORMATION**

\* Name:

Position

\* Organisation

\* Address

\* Country

\* Email

### **SURVEY QUESTIONS (1 to 3)**

\* Question 1: As a prerequisite for the upcoming EFSA baseline survey concerning the prevalence of MRSA in pigs, your laboratory will be expected to conduct MRSA identification. Regarding the identification of MRSA in your laboratory, which method do you intend to apply, PCR or WGS?

- MRSA identification by PCR
- MRSA identification by WGS
- Other (please specify in comments)

Comments:

\* Question 2: The EURL-AR will offer a proficiency test (PT) for PCR (or WGS) identification of MRSA. Does your laboratory intend to participate in such proficiency test?

- We intend to participate in the PT
- We do NOT intend to participate in the PT
- Other (please specify in comments)

**APPENDIX 1 – SIGNUP SURVEY AND QUESTIONNAIRE PAGE 2/2**

Comments:

\* Question 3: Is your laboratory interested in receiving the quality control strains - even if not intending to participate in the above mentioned PT?

- We are interested in receiving the control strains
- We are NOT interested in receiving the control strains
- Other (please specify in comments)

Comments:

**END OF SURVEY**

## 7 APPENDIX 2 TEST FORM

Example of test form for collecting results from the three control strains as well as the ten 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 PCR-1:

Gene name	PCR-1
<i>spa</i>	<input type="checkbox"/>
<i>mecA</i>	<input type="checkbox"/>
PVL	<input type="checkbox"/>
<i>scn</i>	<input type="checkbox"/>
CC398	<input type="checkbox"/>

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 PCR-2:

Gene name	PCR-2
<i>spa</i>	<input type="checkbox"/>
<i>mecA</i>	<input type="checkbox"/>
PVL	<input type="checkbox"/>
<i>mecC</i>	<input type="checkbox"/>

Comment:

\_\_\_\_\_



## 8 APPENDIX 3 EXPECTED RESULTS

### Expected results MRSA PCR control- and test strains

ID Control Strains	Gene:	<i>spa</i>	<i>mecA</i>	<i>mecC</i>	<i>pvl</i>	<i>scn</i>	CC398	MLST	<i>spa</i> -type	MRSA/MSSA
	Product size:	200-600 bp	162	138	85	130	106			
PCR-1-C1 EURL ST-12.7	<i>spa, pvl, scn</i>	+	-	-	+	+	-	ST1	t127	MSSA
PCR-1-C2 EURL ST-11.3	<i>spa, mecA, CC398</i>	+	+	-	-	-	+	ST398	t34	MRSA
PCR-2-C3 EURL ST 17.7	<i>spa, mecC</i>	+	-	+	-	-	-	ST130	t9268	MRSA

ID Test Strains	Gene:	<i>spa</i>	<i>mecA</i>	<i>mecC</i>	<i>pvl</i>	<i>scn</i>	CC398	MLST	<i>spa</i> -type	MRSA/MSSA
	Product size:	200-600 bp	162	138	85	130	106			
EURL MRSA-PT-01	<i>S. aureus</i>	+	-	-	-	-	+	ST398	t34	MSSA
EURL MRSA-PT-02	<i>S. aureus</i>	+	-	+	-	-	-	ST130	t843	MRSA
EURL MRSA-PT-03	<i>S. aureus</i>	+	+	-	-	+	-	ST36	t18	MRSA
EURL MRSA-PT-04	<i>S. epidermidis</i>	-	+	-	-	-	-	ST59	N/A	MRSE
EURL MRSA-PT-05	<i>S. aureus</i>	+	+	-	-	-	+	ST398	t34	MRSA
EURL MRSA-PT-06	<i>S. aureus</i>	+	-	-	+	+	-	ST30	t21	MSSA
EURL MRSA-PT-07	<i>S. aureus</i>	+	+	-	-	-	+	ST398	t4571	MRSA
EURL MRSA-PT-08	<i>S. aureus</i>	+	-	-	-	-	+	ST398	t34	MSSA
EURL MRSA-PT-09	<i>S. aureus</i>	+	-	+	-	-	-	ST4307	t843	MRSA
EURL MRSA-PT-10	<i>S. aureus</i>	+	+	-	-	-	-	ST1412	t917	MRSA