LABORATORY PROTOCOL

MRSA Multiplex PCR-1

PCR AMPLIFICATION OF CC398, MECA, PVL, SCN AND SPA

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Version 1

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based on protocol from the National Reference Laboratory for Antimicrobial Resistance at Statens Serum Institut, Denmark

HISTORY OF CHANGES				
Version	Sections changed	Description of change	Date	Approval
1	New document	Defined as MRSA Multiplex PCR-1	Nov 2022	EURL-AR

Background

This PCR protocol is issued in relation to the preparation of an EU-wide baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in pigs. The objective of the survey is to estimate the MRSA prevalence in fattening pigs at slaughter, and this PCR will be included as a method for confirmation of *Staphylococcus aureus* species (by *spa* gene) and presence of the most dominant MRSA identifier, the resistance gene *mecA*. Further, this PCR will give information about the host association by confirming presence of identifiers for Panton-Valentine Leucocidin (PVL), *scn* and CC398. Presumptive MRSA isolates, which are negative for *mecA*, can additionally be screened for *mecC* by the MRSA Multiplex PCR-2, also available on the EURL-AR website.

Purpose

The purpose of the protocol is to screen presumptive methicillin-resistant *Staphylococcus aureus* (MRSA) for the presence of genes encoding methicillin-resistance (*mecA*), relation to human (*scn*) or pigs (CC398), Panton-Valentine Leucocidin (PVL) and amplification of Protein A (*spa* gene) for sequencing and *spa*-typing. Isolates positive for the CC398 identifier, does not require additional *spa*-typing The method we recommend and describe below was first described (in part) by Stegger *et al,* 2012 and by Rasmussen *et al*, 2019.

This PCR method is referred to as the PCR-1 in the "Technical specifications for a baseline survey on the prevalence of methicillin-resistant Staphylococcus aureus (MRSA) in pigs" (EFSA, 2022).

Protocol

Preparation of DNA-templates/DNA extraction using boiled lysates

- Grow *S. aureus* strains on 5% blood agar and incubate at 35 °C for 16-18 hrs
- Add 200 µl PCR grade H_2O per tube
- Suspend 3-4 colonies (~ 1 µl)
- Incubate at 96 °C for 10 min
- Centrifuge at 4500×*g* for 3 min

The template is ready for use or store DNA samples at -20°C*.

* Vortex and centrifuge the DNA suspension (13200 rpm for 5 min), before use.

PCR controls:

Two suggested positive control strains for this PCR:

PVL+ *scn* + *spa:* *S. aureus* EURL-ST-12.7 (EQAS 2018)

***mecA* + CC398 + *spa*:** *S. aureus* EURL-ST-11.3 (EQAS 2017)

These control strains have previously been distributed to the EURL-AR network laboratories as EQAS strains in 2017 and 2018, and can be acquired from the EURL-AR on request.

Preparation of primers *spa/ mecA/* PVL */scn/* CC398:

The following primers are used in this multiplex PCR setup:

Primer list MRSA multiplex PCR-1:

Primer name	Primer # (EURL-AR)	Sequence
spa-1113F	2819	5' – TAAAGACGATCCTTCGGTGAGC – 3'
spa-1514R	2820	5' – CAGCAGTAGTGCCGTTTGCTT – 3'
mecA P4	2821	5' – TCCAGATTACAACTTCACCAGG – 3'
mecA P7	2822	5' – CCACTTCATATCTTGTAACG – 3'
PVL-F	2823	5' – GCTGGACAAAACTTCTTGGAATAT – 3'
PVL-R	2824	5' – GATAGGACACCAATAAATTCTGGATTG – 3'
*scn*F1	3240	5'– TACTTGCGGGAACTTTAGCAA-3'
*scn*R1	3241	5'– AATTCATTAGCTAACTTTTCGTTTTGA-3'
FP2sau 1	3242	5'– GAGAATGATTTTGTTTATAACCCT AG-3'
CC398r1	3243	5'– CAGTATAAAGAGGTGACATGACCC CT-3'

Prepare forward and reverse primer-mix individually:

Primer-mix 1 *spa-*1113F*/ mecA* P4*/* PVL-F*/ scnF1/ FP2sau1* <u>Forward</u> primers:

- Take 900 µL H_2O
- Add 20 µL *spa*-1113F (100 µM)
- Add 20 µL *mecA* P4 (100 µM)
- Add 20 µL PVL-F (100 µM)
- Add 20 µL *scnF1* (100 µM)
- Add 20 µL *FP2sau 1* (100 µM
- Vortex s*pa/mecA P4 /*PVL-F/scnF1/FP2sau1 mix

Primer-mix 2 *spa-*1514R*/ mecA* P7*/* PVL-R*/ scnR1/ CC398 r1* <u>Reverse</u> primers:

- Take 900 µL H_2O
- Add 20 µL *spa*-1514R(100 µM)
- Add 20 µL mecA P7 (100 µM)
- Add 20 µL PVL-R (100 µM)
- Add 20 µL *scnR1* (100 µM)
- Add 20 µL *CC398 r 1* (100 µM)
- Vortex spa/mecA P7 /PVL-R/scnR1/CC398 r1 mix

Dilution of *spa* primers for sequencing

To obtain *spa* primers of 10µM:

- Add 100µl *spa*-1113F (100µM) to 900µl of PCR H_2O
- Add 100µl *spa*-1514R (100µM) to 900µl of PCR H_2O

Sample preparation for PCR

Reaction mix

QIAGEN Multiplex 2X PCR kit or as an alternative, EURL-AR suggests (in PCR-2) the use of Master mix (DreamTaq™ Green PCR Master Mix) to facilitate the PCR reaction preparation, by including loading buffer, allowing for direct loading on electrophoresis gel after PCR amplification.

The setup and running conditions are also described in the PCR-1 Sample sheet (page 7) which contains PCR mix, control strains and conditions.

Template:

As template for the PCR, we recommend to use 2 µl of a 10x dilution of the DNA extractions or lysates in a 25 µl PCR reaction.

PCR Program

1 CYCLE	30 CYCLES	1 CYCLE
94 ˚C 15 min.	94 ˚C 30 sec.	72 ˚C 10 min.
	59 ˚C 60 sec.	Hold ~ 5 ˚C
	72 ˚C 60 sec.	

Electrophoresis:

2 % Agarose gel

Run 5-8 µl of the PCR products (you do not need to mix loading buffer for the electrophoresis in case you use the DreamTaq Green Master mix). Run in parallel with 10µL of a 100 bp Ladder molecular weight marker on a 2 % agarose gel in 0.5X TBE buffer with e.g. SYBR safe stain (used as an alternative to Ethidium Bromide Cf. PCR-2). Run electrophoresis for 1 hr at 110V.

Alternative: 2% E-gel

5-10 µL PCR product is added to 10-15 µL PCR H2O. 20 µL of the mixture is loaded in the wells. 10µL of 100 bp DNA ladder is run parallel with the PCR products.

Gel photo

Take a picture in the transilluminator under UV light. Observe the bands and interpret the results according to the description below and the figure (Figure 1).

The presence of *spa, mecA, scn*, CC398 and PVL genes is checked.

- *mecA* - the *mecA* fragment to be amplified has expected size of 162 bp
- *scn –scn* fragment to be amplified has an expected size of 130 bp
- CC398 - CC398 fragment to be amplified has an expected size of 106 bp
- PVL - the PVL fragment to be amplified has an expected size of 85 bp

- *spa* - the *spa* fragment resulting from the amplification is variable in size and ranges from 180-600 bp depending on the *spa* type present and this fragment should be amplified from all *S. aureus* strains (no amplification of the *spa* fragment indicates the isolate is not a *S. aureus* and further identification procedures might be necessary to determine the species, in case this is necessary)

Note: The *spa* PCR product from the multiplex PCR can be cut out from the gel, purified and used for sequencing of the *spa* fragment for *spa* typing, directly.

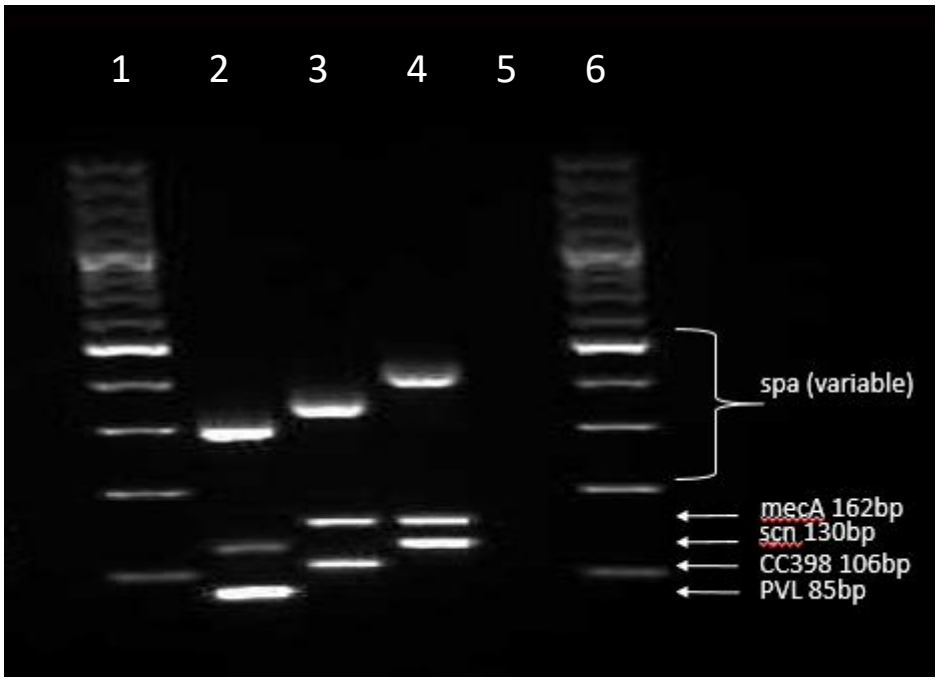

Figure 1. Multiplex PCR for detection of *spa*, *mecA*, *scn,* CC398 and PVL

Lane 1: 100-bp ladder (GeneRuler)

Lane 2: MSSA: *S. aureus* EURL-ST-12.7 (*spa,* PVL(*lukF-PV*) and *scn*)

Lane 3: MRSA: *S. aureus* EURL-ST-11.3 (mecA + CC398)

Lane 4: MRSA: *S. aureus* 50A2047 (*spa, mecA* and *scn*; alternative control from PCR-2)

Lane 5: negative control (H_2O)

Lane 6: 100-bp ladder (GeneRuler)

References:

Stegger M, Andersen PS, Kearns A, Pichon B, Holmes MA, Edwards G, Laurent F, Teale C, Skov R, Larsen AR. (2012) Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue *mecA(LGA251)*. Clin Microbiol Infect. 2012 Apr;18 (4):395-400. https://doi.org/10.1111/j.1469-0691.2011.03715.x

Rasmussen SL, Larsen J, van Wijk RE, Jones OR, Berg TB, Angen Ø, Larsen AR. (2019) European hedgehogs *(Erinaceus europaeus)* as a natural reservoir of methicillin-resistant *Staphylococcus aureus* carrying *mecC* in Denmark. PLoS ONE 14(9): e0222031. https://doi.org/10.1371/journal.pone.0222031

European Food Safety Authority (EFSA), Aerts M, Battisti A, Hendriksen R, Larsen J, Nilsson O, Abrahantes JC, Guerra B, Papanikolaou A, Beloeil PA (2022) Technical specifications for a baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in pigs. EFSA Journal Volume 20, Issue10, October 2022. https://doi.org/10.2903/j.efsa.2022.7620

PCR-1 Sample sheet (Example for setup)

Primer 1: Primer mix containing: 2819-2821-2823-3240-3242
Primer 2: Primer mix containing: 2820-2822-2824-3241-3243
DNA polymerase: DreamTaq™ Green PCR Master Mix
PCR products: *spa* (variable: 200-600 bp); *mecA* (162 bp); *scn* (130bp); CC398 (106bp), PVL (~85bp)
PCR-controls: MSSA: EURL-ST-12.7 MRSA: EURL-ST-11.3 Additional control strain: MRSA: *S. aureus* 50A247 (from MRSA PCR-2) **Remark: DNA template can be diluted 10X if necessary and 2 µl of the diluted DNA be used as template**

Number of samples	1	
PCR H_2O	5,5	**0**
2xGreen PCR Master Mix	12,5	**0**
dNTP	0	**0**
25 mM $MgCl_2$	0	**0**
Primer 1 (0,5 of each)	2,5	**0**
Primer 2 (0,5 of each)	2,5	**0**
Taq polymerase	0	**0**
Total volume	23	**0**

1. 5 min at 94 °C
2. 30 Cycles
   - 30 Sec. at 94 °C
   - 1 Min. at 59 °C
   - 1 Min at 72 °C
3. 10 min at 72 °C
4. _____ hold at 5 °C

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