Laboratory Protocol

Isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) from food-producing animals and farm environment

April 2023
Version 3

Written by EURL-AR

HISTORY OF CHANGES				
Version	Sections changed	Description of change		Approval
3	1.3	The number of days between the collection of samples and the start of the analysis was updated from three to seven.	11-04-2023	EURL-AR
2	Background 1.1 – 1.2 2.1 Appendix 2	Added: "*In 2022, the EURL-AR modified method was reviewed and slightly updated preparing its use for the future MRSA baseline survey – now scheduled for 2025.*" Sample preparation and pooling procedure was updated to meet the latest guidelines from the EU legislation. In the enrichment step, MHB volumes were updated. Pictures of MRSA colony morphology on different media (Appendix 2) were updated.	31-01-2023	EURL-AR
1	New document Changed to be from animals and farm environment	This document replaces previous EURL-AR protocol. The main difference is the exclusion of the second enrichment step with cefoxitin and aztreonam. Editorial changes were also performed.	17 Apr 2018	Valeria Bortolaia, Rene Hendriksen
0	Dust protocol	Original protocol.	2009	Lina Cavaco

Background

The most common method for detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in samples from food-producing animals and farm environment (e.g., nasal, skin, and dust swabs) consists of a pre-enrichment step followed by a selective enrichment step before plating on chromogenic MRSA-selective and -indicative agar (1). A recent study at two laboratories has, however, shown that inclusion of the selective enrichment step leads to a higher number of false-negative results when analysing nasal and skin swabs from pigs and environmental swabs from pig stables than the same procedure omitting this stage (2). Studies in poultry (layers and broilers) and cattle did not detect significant differences in the performance of the two methods (3,4). Based on these findings, the EURL-AR modified the method for isolation of MRSA from samples from food-producing animals and farm environment. In 2022, the EURL-AR modified method was reviewed and slightly updated preparing its use for the future MRSA baseline survey – now scheduled for 2025.

References

1. European Food Safety Authority (EFSA). Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in methicillin-resistant *Staphylococcus aureus* in food-producing animals and food. EFSA J. **2012;** 10(10):2897. https://www.efsa.europa.eu/en/efsajournal/pub/2897

2. Larsen J, Sunde M, Islam MZ, Urdahl AM, Barstad AS, Larsen AR, Grøntvedt CA, Angen Ø. Evaluation of a widely used culture-based method for detection of livestock-associated meticillin-resistant *Staphylococcus aureus* (MRSA), Denmark and Norway, 2014 to 2016. Euro Surveill. **2017;** 22(28): pii=30573. http://www.eurosurveillance.org/content/10.2807/1560-7917.ES.2017.22.28.30573

3. Nemeghaire S, Roelandt S, Argudín MA, Haesebrouck F, Butaye P. Characterization of methicillin-resistant *Staphylococcus aureus* from healthy carrier chickens. Avian Pathol. **2013**;42(4):342-6.

4. Nemeghaire S, Argudín MA, Haesebrouck F, Butaye P. Epidemiology and molecular characterization of methicillin resistant *Staphylococcus aureus* nasal carriage isolates from bovines. BMC Vet Res. **2014**;10(1):153.

5. EFSA technical specifications for a baseline survey on the prevalence of MRSA in food-producing animal populations (https://www.efsa.europa.eu/en/efsajournal/pub/7620).

Contents

Procedure		Theory/comments
1. Sample collection and storage		
1.1 For the MRSA monitoring, member states should collect nasal samples taken at slaughterhouse from fattening pigs, according to the draft Commission Implementing Decision (EU) 2020/1729 and the latest recommendations by EFSA about sampling design and sample types, as regards monitoring of MRSA in fattening pigs.		Always refer to the latest recommendations by EFSA for sampling design and sample types to be collected for MRSA monitoring (https://www.efsa.europa.eu/en/efsajournal/pub/7620).
1.2 Twenty nasal swabs from twenty different pigs shall be pooled into four composite groups of five samples for the isolation procedures.		If the epidemiological unit comprises of less than twenty pigs, all the pigs of this epidemiological unit shall be sampled, and the resulting samples shall be pooled as evenly as possible to form the four composite groups of samples.
1.3 It is recommended to store swab samples at 4-8 °C to avoid condensation, which may occur on refrigeration. Moreover, it is recommended to initiate analysis i) within seven days of collection for qualitative purposes (i.e., for scoring samples as positive/negative); and ii) on the same day of collection or, if not possible, one day after collection for quantitative purposes.		There is limited knowledge on the effect of duration and temperature of storage of different sample types on MRSA viability and counts. Different commercially available transport media ensure viability of bacteria at room temperature for different amount of times. Room temperature, however, varies greatly across seasons and EU countries, which might lead to results that are not comparable across EU. As the purpose of harmonised protocols is to ensure comparability across EU countries, samples should be kept at comparable conditions.
2. Sample analysis		**IMPORTANT:** the present protocol was validated with nose, skin and dust samples from pigs and pig stables in countries with high and low prevalence of livestock-associated (LA)-MRSA (Larsen *et al.* 2017). As sensitivity and specificity of detection methods might be affected by sample type, animal species of origin and within-sample MRSA prevalence, laboratories are invited to contact the EURL-AR for sharing their experiences with this and/or other MRSA isolation protocols, with the overall aim to ensure the highest sensitivity and specificity of MRSA detection in samples from food-

		producing animals, the farm environment and food across EU.
2.1 Cover the samples in Mueller-Hinton broth containing 6.5% sodium chloride (NaCl) and incubate at 35-37°C for 16-24 h.		This step selects for staphylococci and other salt-tolerant bacteria. For this validation protocol, the following volumes of Mueller-Hinton broth containing 6.5% sodium chloride (NaCl) were used: • 10 mL for pools of five cotton swabs • 500 mL for pools of two to three cloth swabs • 300 mL for individual cloth swabs • In case ESwabs are used, it is recommended to transfer 100 µL of Liquid Amies medium from the transport tube into 5 mL of MHB containing 6.5% NaCl. For single dry swabs, it is recommended to place the dry swab in 5-10 mL of MHB containing 6.5% NaCl, depending on the tube size used, ensuring that the swab is covered by the liquid. The effect of using different volumes is unknown.
2.3 Spread a 10-µl loopful of the broth on *Brilliance* MRSA 2 agar (Oxoid) and incubate at 35-37°C for 16-24 h.		Presumptive MRSA appear as denim blue colonies after overnight incubation.
2.4 Subculture presumptive MRSA colonies on blood agar and incubate at 35-37°C for 22-24 h.		MRSA colonies on blood agar are greyish or yellowish and usually surrounded by a zone of haemolysis. The catalase test can be used to distinguish staphylococci from enterococci, which sometimes produce similar colony morphology on *Brilliance* MRSA 2 agar. See Appendix 2 for pictures of MRSA colony morphology on different media.
3. Identification of MRSA		
3.1 Confirm presumptive MRSA colonies by PCR-1 and/or PCR-2, according to the protocols described in the documents entitled "MRSA multiplex PCR-1 protocol; PCR amplification of CC398, *mecA*, PVL, *scn* and *spa*" and "MRSA multiplex PCR-2 protocol; PCR amplification of *mecA*,		MRSA isolates are positive for *spa* and either *mecA* or *mecC*, whereas the presence of *pvl* is variable. MRSA-confirmed isolates can be further *spa*-typed in order to determine the corresponding Clonal Complex (CC). Isolates for which no CC can be inferred from the *spa*-type should be further typed

mecC, PVL and *spa"* on the EURL-AR website.		by MLST-typing. These methods are described in the documents entitled "*spa*-typing protocol" and "MLST typing" on the EURL-AR website.

APPENDIX 1

Composition and preparation of culture media and reagents

The media and reagents are available from several companies including Oxoid, BD, Merck and Difco. The composition of the dehydrated media given below is an example and may vary among the different manufacturers. Also, the media should be prepared according to the manufacturer's description if that differs from the description given here.

Mueller Hinton Broth with 6.5% NaCl

Formula	g/L
Dehydrated beef infusion	300
Casein hydrolysate	17.5
Starch	1.5
Sodium Chloride	65
pH 7.3 +/- 0.1	

Oxoid *Brilliance*™ MRSA 2 Agar
Prepare according to the manufacturer's description.

Blood Agar

Formula	g/L
"Lab-Lemco" powder	10.0
Neutralised peptone	10.0
Sodium chloride	5.0
Agar	15.0
pH 7.3 +/- 0.2	

After cooling to 50°C, add 7% of defibrinated sheep or horse blood.

APPENDIX 2

Pictures showing typical colony morphology of MRSA on Oxoid *Brilliance*™ MRSA 2 Agar and Blood Agar

Typical growth of MRSA and MSSA on Oxoid *Brilliance*™ MRSA 2 Agar and Blood Agar are presented in Figure 1, Figure 2, and Figure 3. MSSA strains could develop a few colonies on Oxoid *Brilliance*™ MRSA 2 Agar (Figure 4), however this should be considered a negative result. MRSA develops similar growth levels on Oxoid *Brilliance*™ MRSA 2 Agar as on blood agar (Figure 1).

Figure 1. Typical pictures of MRSA on Oxoid Brilliance™ MRSA 2 Agar (Left) and Blood Agar (Right). MRSA colonies develop a denim blue colour on Oxoid Brilliance™ MRSA 2 Agar and reach growth levels similar to growth on blood agar.

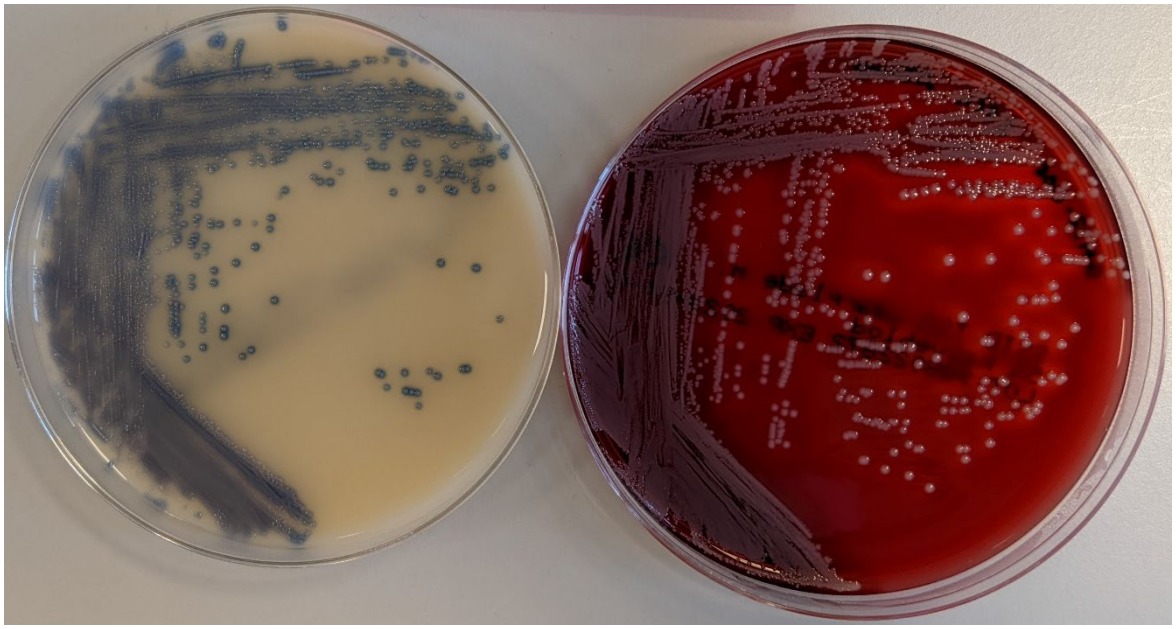

Figure 2. Denim blue colonies of MRSA growing on Oxoid Brilliance™ MRSA 2 Agar.

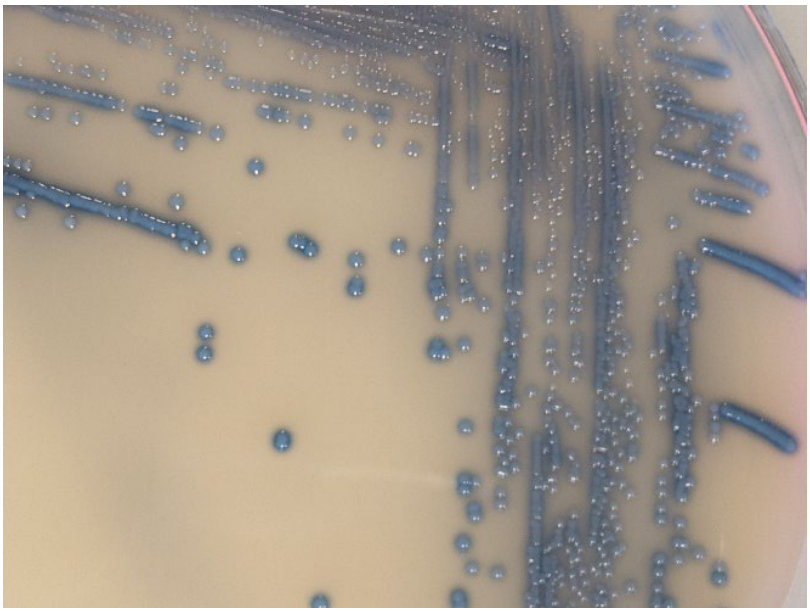

Figure 3. Typical growth of MSSA on Oxoid Brilliance™ MRSA 2 Agar (Left) and Blood Agar (Right). MSSA does not grow on Oxoid Brilliance™ MRSA 2 Agar.

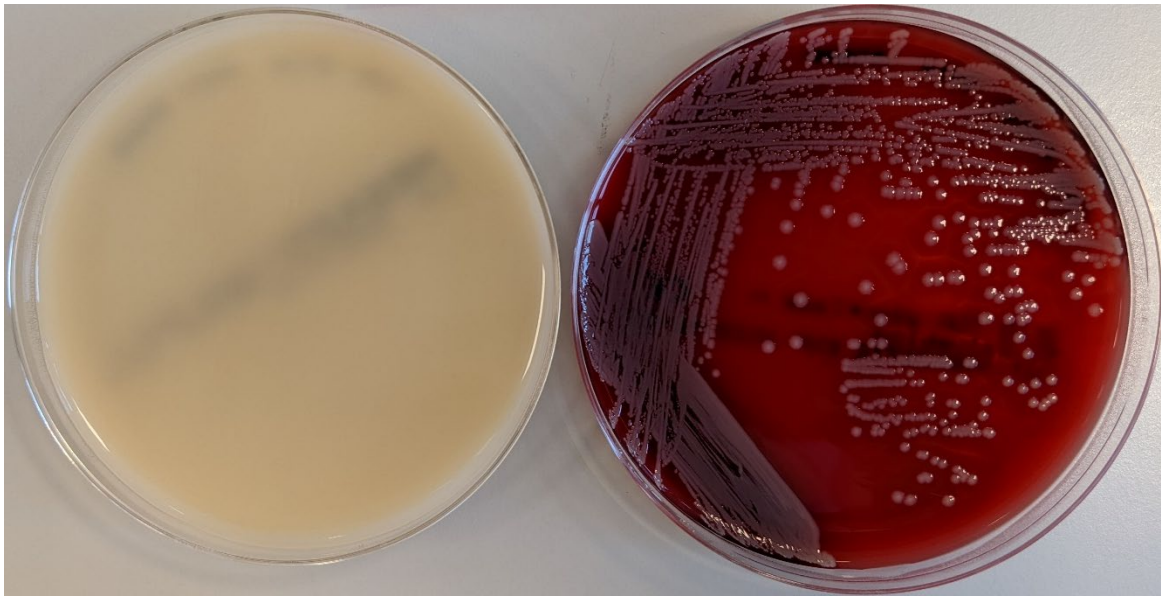

Figure 4. A few MSSA colonies growing on Oxoid Brilliance™ MRSA 2 Agar.

APPENDIX 3
Flow Diagram

Isolation of MRSA from samples from food-producing animals and their environment

Enrichment
Cover the samples in Mueller-Hinton broth containing 6.5% sodium chloride (NaCl)
(35-37°C, 16-24 h)

↓

Isolation
Spread a 10-µl loopful of the broth on *Brilliance* MRSA 2 agar (Oxoid)
(35-37°C for 16-24 h)

↓

Sub-culture
Subculture presumptive MRSA colonies on blood agar
(35-37°C for 22-24 h)

↓

Identification
Multiplex PCR-1 for detection of *mecA*, *scn*, *spa* and *pvl.*
If *mecA* negative, Multiplex PCR-2 can be applied to test for *mecC*