Report of the 5th External Quality Assurance System on Identification and Typing of Methicillin resistant *Staphylococcus aureus* (MRSA), 2013

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1. Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) have been a concern in the recent years due to their emergence in livestock and the possible occupational hazards for humans in close contact with animals. In 2008, the European Commission (EC) took a decision of screening the population of pig farms in Europe for this new emerging pathogen, taking this screening into the framework of the baseline screening for *Salmonella* in pig farms (European Commission Decision 2008/55/EC). This screening has been performed in most European Countries by laboratories appointed by the national authorities and according to a common protocol defined by the EC.

The European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR) took part in the development and support of the laboratories by providing assistance in setting up the methods for isolation, identification, and typing of MRSA. Furthermore, the EURL-AR provided training in the specific methods on individual basis as well as in an MRSA training course that was organized in April 2009.

In June 2009, the EURL-AR in addition to the External Quality Assurance System (EQAS) already performed, launched an MRSA specific EQAS, to enhance the capacity of the laboratories in MRSA isolation, identification and typing. This EQAS aimed at the identification of potential problems and identification of focus areas for training/education that might improve data quality in future European studies focusing on MRSA epidemiology.

Initially, the first trials were aimed at isolation of MRSA from animal-related sources, such as dust isolates and swabs.

The methods used in this EQAS reflect the methods recommended by EFSA technical specifications on the Scientific Report published by EFSA in 2012 on MRSA isolation, detection and typing methods (EFSA, 2012).

In 2013, we launched the fifth EQAS performed on MRSA which included identification and typing of MRSA, and was based on pure isolates. Unlike the previous EQAS, the processing of isolates did not include isolation steps but identification of MRSA by molecular methods, species identification and detection of methicillin resistance (*mecA* and *mecC* gene) were mandatory items for confirmation of MRSA. An optional sequence based typing module based on *spa* typing was also offered. The *spa* typing trial was offered to increase capacity of laboratories in MS for performing standard MRSA typing using a method which is easily performed, and which results are robust and comparable to assess the epidemiology of MRSA in Europe.

At this point, no mandatory surveillance for MRSA is defined by the European Commission. It is, however, considered relevant to enhance and/or maintain the objectives of this EQAS, i.e. the laboratories should have the methods implemented and ensure their performance. Therefore, this EQAS is aimed at preparing the laboratories for correctly performing the identification and molecular detection of MRSA and maintaining this capacity at MS level.

No thresholds have been set in advance to evaluate the performance of the participating laboratories, or to classify the results of this EQAS.

The MRSA EQAS was organized by the National Food Institute (DTU Food), Kgs. Lyngby, Denmark, and the verification/confirmation of the strains used in

the preparation of the test isolates was performed at the Food and Drug Administration Laboratory in the US (FDA).

The data in this report are presented with laboratory codes. A laboratory code is known to the individual laboratory, whereas the entire list of laboratories and their codes is confidential and known only to the EURL-AR and the EU Commission. All conclusions are public. The technical advisory group for the EURL-AR EQAS scheme consists of competent representatives from all National Reference Laboratories for Antimicrobial Resistance (NRLs-AR), who meet annually at the EURL-AR workshop.

2. Materials and Methods

2.1 Participants in EQAS 2013

A pre-notification (App 1), inviting the participants to the EQAS for antimicrobial susceptibility testing of Staphylococci, Enterococci and *E. coli* and for the MRSA EQAS 2013 was issued by e-mail to the EURL-AR network on the 15th of April 2013 to the NRL-AR's. Additionally, the laboratories that participated in the MRSA baseline studies in 2008 and participated in the MRSA EQAS in the previous year were invited to participate using the same pre-notification.

All participants were included in a participant list (App 2) before the preparation and shipping of the isolates. Participation was free of charge but each laboratory was expected to cover expenses associated with their own analysis.

2.2 Preparation of isolates for shipment

Ten stab cultures containing *Staphylococcus* spp were prepared for identification of species, detection of methicillin resistance, and typing. The isolates were either methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *S. aureus* (MSSA), methicillin resistant or susceptible *S. pseudintermedius* (MRSP or MSSP) and/or coagulase negative staphylococci strains (CNS). Eight of the test isolates selected for the MRSA EQAS were the same isolates as those to be used in the EQAS for antimicrobial susceptibility testing of *S. aureus* and two were additionally prepared for this EQAS, exclusively (EURL-ST 7.9 and ST 7.10). The isolates were prepared in advance and subcultured in weeks 21-23 before agar stick preparation. Homogeneity tests were performed for ensuring the purity of the agar stick cultures, and MRSA identification and antimicrobial susceptibility profiles were confirmed.
The strains used for the EQAS were selected from the strain collections at DTU-Food and the identifications were confirmed by PCR and 16S rDNA and/or *spa* detection and sequencing. Furthermore, the *spa* types of the MRSA isolates were determined by PCR and sequencing and assigned according to the *spa* server using Bionumerics (App 3). Identification and the *spa* type of the selected isolates were verified and confirmed by parallel analysis conducted at FDA in the US. 2.3 Identification of MRSA

The protocol for identification of the MRSA isolates was made available on the EURL-AR website (http://www.eurl-ar.eu) (App.4). The protocol for identification of methicillin resistance recommended was the same as in 2012 based on a multiplex PCR able to detect both *mecA* and *mecC* genes and to simultaneously amplify fragments of the *spa* and *pvl* genes (Stegger *et al.,* 2012).

2.4 *spa* typing

The isolates used for this EQAS were typed by

a single locus sequence typing method - *spa* typing (Shopsin *et al.* 1999) and in addition the *spa*-types were verified at the FDA.

The laboratories with capacity to perform *spa* typing were invited to submit the *spa* typing results to the MRSA EQAS database, which were evaluated for accuracy against the expected *spa* types.

2.5 Distribution

The agar sticks containing stab cultures were kept at 4°C between the preparation and shipment process (2-4 weeks). The tubes containing the cultures in nutrient agar were enclosed in double pack containers (class UN 6,2) and sent to the selected laboratories according to the International Air Transport Association (IATA) regulations as "Biological Substance category B" classified UN3373. The parcels were dispatched from DTU-Food on June 17th, 2013.

2.6 Procedure

The laboratories were instructed to download the protocol and test forms (App. 4 and 5), from http://www.eurl-ar.eu

The EQAS protocol was based on the method recommended by the EURL-AR for the identification of the MRSA isolates using a multiplex PCR which would allow identification of *S. aureus* and also identify the *mecA* and *mecC* genes, mandatory for the classification of isolates as MRSA, Regarding *spa* typing the standard recommended *spa* typing procedure was recommended (App 4).

After completion of the tests, the laboratories were requested to enter the obtained results into an electronic record sheet in the EURL-AR web based database through a secured individual login, or alternatively send the record sheets by fax to DTU Food (App 5). The database was activated on the June 21st, 2013 and closed after the deadline on September 10th, 2013 and the evaluation reports were made available by this date.

The isolates were categorised as positive or negative in function of the identification of MRSA isolates. Negative isolates could be subdivided into different categories (negative isolate, MSSA, MRCNS, MRSP, CNS), however, they would be grouped as negative. Final interpretations of the results obtained were compared to the expected results and classified as correct or incorrect without further classification of the deviation.

As an optional item, results of *spa* types were requested for typing the positive MRSA isolates. In this case, the participants would be invited to choose the obtained *spa* type from a pick list, and the typing result would be evaluated against the expected *spa* type and classified into correct or incorrect.

3. Results

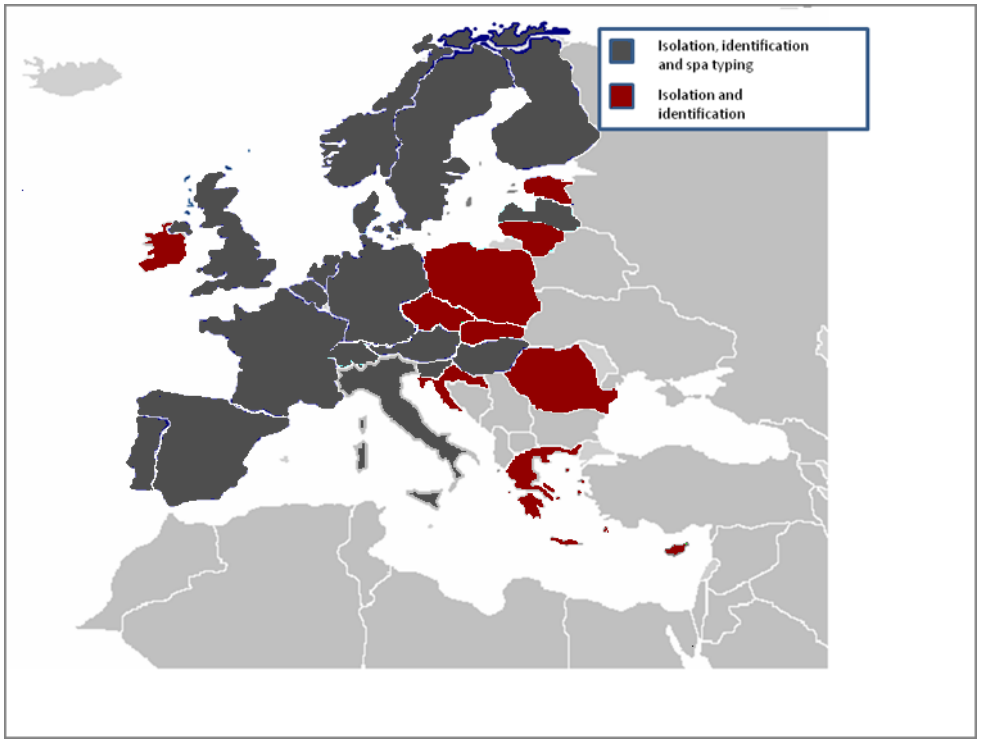
A total of 34 laboratories; including 26 NRLs, six additional non-NRLs in EU and laboratories from Norway and Switzerland responded to the pre-notification, and were enrolled in the EQAS.

When the deadline for submitting results was reached, 32 laboratories in 26 European countries (including Switzerland and Norway) had uploaded data. Two of the laboratories decided not to take part due to technical issues (Lab #39 and #40).

One laboratory or more from each of the following countries provided results to the EQAS (Figure 1): Austria, Belgium, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Lithuania, the Netherlands, Norway, Poland, Portugal, Romania, Slovakia,

Slovenia, Spain, Sweden, Switzerland, and UK.

Figure 1. Participating countries in MRSA EQAS 2013. Participating countries are marked in green for laboratories participating in MRSA identification.Dark blue represents countries participating in the *spa* typing as well



3.1 Methods used by EQAS-participants

Of the 32 laboratories submitting results, all (100%) of the laboratories participated in the MRSA identification module whereas only 17 laboratories (53%) participated in the optional *spa* typing module offered.

Information on the methods used for isolation, identification and typing was collected from the participants through the database.

The confirmatory testing of *S. aureus* was performed using the current EURL-AR recommended PCR-protocol containing the *mecA*, *mecC*, *pvl* and *spa* gene in most laboratories uploading results (n=21) and the other referred methods for identification included the former EURL-AR-recommended method (n=3), other published (n=2) or in-house PCR and RT-PCR methods (n=2). The remaining laboratories did not reply to this question.

The species identification was mostly performed by PCR, using the EURL-AR recommended PCR primers (n=14), however, six laboratories used the former protocol for this purpose. Other methods included using other published or in-house PCR methods in the remaining laboratories that reported methods (n=3). As additional identification methods, some participating laboratories added *nuc* and fe*m*B PCR, coagulase tests, biochemical tests, Vitek and MALDI-TOF identification systems to

confirm the species ID for some or all of the test strains.

Table 1. The overall performance of MRSA identification, 2013.

Identification of MRSA isolates		Correctly classified isolates	
Number of performed tests		Number of correct tests N(%)	
n	%	N	%
314	100	308	98%
Number of expected negative tests		Number of correctly identified negative tests	
n	%	N	%
122	40%	119	98%
Number of expected positive tests		Number of correctly identified positive tests	
n	%	N	%
192	60%	189	98%

3.2 MRSA identification

A total of 314 tests, were performed and the overall result indicate that 308 tests were correct, corresponding to 98%. From the 122 tests performed on the four strains expected to be negative, 98% (n=119) were correctly assigned, and the remaining three were found false positive. Regarding the 192 tests performed on the six strains expected to be found positive, also 98% of these were correctly found positive (n=189) (Table 1).

The results per strain show that deviations were observed for strains ST 7.1, 7.4, 7.6, 7.8, 7.9 and 7.10 and accounting one deviation for each strain.

An internal control was prepared in order to have one strain in common to former MRSA EQAS trials (2009, 2010 and 2012). The internal control was isolate EURL-ST 7.4 (MRSA, *spa* t075).

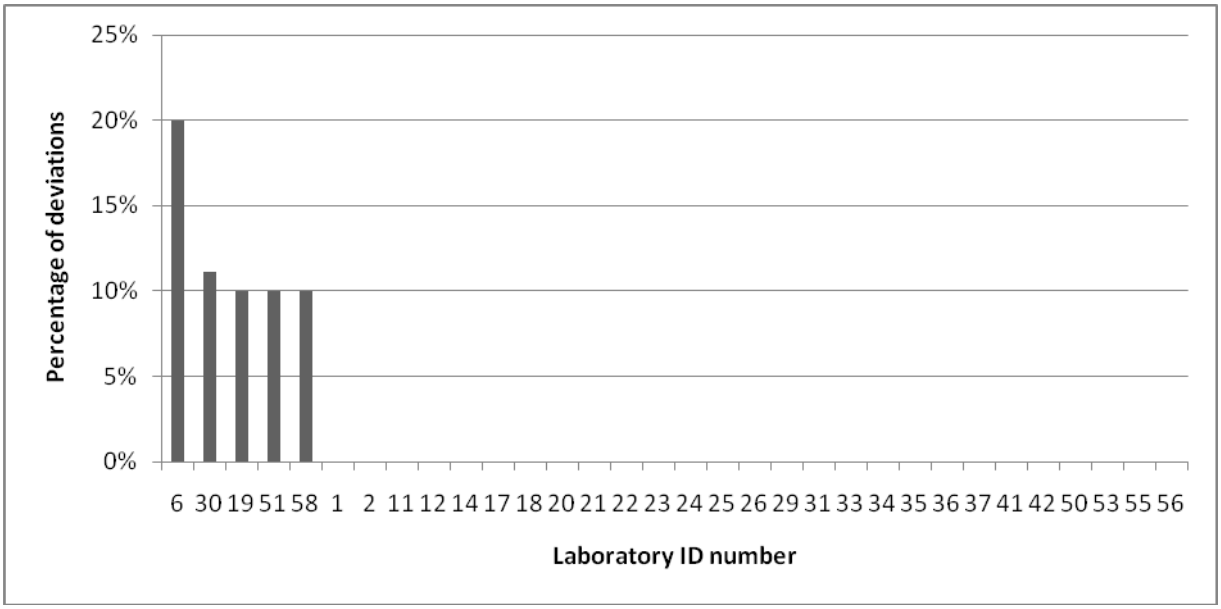
The individual laboratory results provided data for all strains (except for Lab #12 that provided results for seven strains and Lab #30, #31 and #35 that provided results for nine of the strains, leaving out ST 7.10) In general, the test results of this MRSA EQAS showed a very good sensitivity and specificity by most laboratories, indicated by the general accuracy of the tests at 98%. Overall, only three false negatives and three false positives were observed.

The five laboratories obtaining deviations had one or two deviations among the ten isolates shipped (Graph 1).

The laboratory showing the largest number of deviations (Lab #6) failed to identify the MRSA carrying the *mecC* gene (ST 7.1) and considered strain ST 7.10 (*S. pseudintermedius*) to be MRSA. These mistakes (20% deviation) might be explained by the method used for identification and confirmation of MRSA (the former PCR method) which did not detect the *mecC* gene or the *spa* gene. Detection of these genes would distinguish strain ST 7.10 from *S. aureus* species. Furthermore, this laboratory is not performing any surveillance at the MS level for MRSA, and did not previously participate in this EQAS.

Regarding the remaining laboratories that

obtained deviations, they all had one deviation each. In the case of lab #30 the laboratory had 11% deviations (one out of nine results uploaded) and the deviation observed was caused by a false positive result for strain ST 7.6 which was an expected MSSA. The deviations found by lab #19 and #51 were false negative results caused by misidentification of ST 7.4 and ST 7.8 which were both MRSA, identified as CNS. Finally, the deviation obtained by lab #58 was due to the misidentification of ST 7.9 as MRSA where CNS was expected.



Graph 1: Number of deviations per laboratory for identification of methicillin resistant *S. aureus* (MRSA)

3.3 *spa* typing of the MRSA strains

In the *spa* typing module, 17 laboratories participated by uploading *spa* typing data.
The reported results for *spa* typing included 99 tests and the general results show that 98 (99%) of the results were considered correct, whereas one (1%) deviation was observed.

In this module, isolates ST 7.1, ST 7.3, ST 7.4, ST 7.5, ST7.7 and ST 7.8 were expected to be identified positive for MRSA whereas the remaining negative strains ST 7.2, ST 7.6, ST 7.9 and ST 7.10 were either MSSA or non-*S. aureus* and were therefore not expected to be *spa* typed (Table 2).

In this way, the range of uploaded *spa* types diverged from one to ten strains uploaded by each participating laboratory but only the *spa* typing results corresponding to the six expected MRSA strains were evaluated.

Sixteen laboratories had no deviations, and only one laboratory had one deviation (lab #1) due to incorrect *spa* type assignment; t127 was reported, whereas t075 was expected.

Additionally, eleven laboratories uploaded results of *spa* typing for MSSA strains ST 7.2 and ST 7.6, in a total of 22 tests which, if evaluated, were correct *spa* types (n=19). This

indicates that the protocol was probably unclear and could be misunderstood by the participants. One laboratory (Lab #30) uploaded an incorrect *spa* type for MSSA strain ST 7.6 (t2582), which was probably due to a switch in strains with ST 7.5 as the same laboratory incorrectly identified this strain as MRSA.

For the non-*S. aureus* strains, ST 7.9 and 7.10, 11 and 8 laboratories, respectively, submitted the result “not applicable”

Table 2. Results of the *spa* typing trial per isolate.

Isolate number	N participating laboratories	Repeat succession	expected *spa* type	correct	Deviating results (number of deviations)
EURL-ST 7.1	17	04-82-17-25-17-25-25-16-17	t843	17	None
EURL-ST 7.2	12	N/A	N/A (t127)	N Ev	-
EURL-ST 7.3	17	08-16-02-25-02-25	t1250	17	None
EURL-ST 7.4	17	11-19-21-21-12-21-17-34-24-34-22-25	t075	16	t127 (1)
EURL-ST 7.5	16	08-16-02-25-02-25-02-25-34-24-25	t2582	16	None
EURL-ST 7.6	10	N/A	N/A (t3855)	N Ev	-
EURL-ST 7.7	16	08-34-24-25	t4571	16	None
EURL-ST 7.8	16	08-16-02-25-24-25	t108	16	None
EURL-ST 7.9	8	N/A	N/A	N Ev	-
EURL-ST 7.10	11	N/A	N/A	N Ev	-

N/A- not applicable
N Ev- not evaluated

4. Discussion

4.1 MRSA identification

In the 2013 EURL-AR MRSA EQAS trial, the strains were sent as agar stab cultures for the first time, and therefore the isolation part of the trial performed in the former years was not included.

The results were overall very good and both expected MRSA and non-MRSA were mostly well classified, with few exceptions of three false negative and three false positive results, leading to 98% accuracy, overall. One of the laboratories showed 20% deviation. This might be explained by the use of a method differing from the recommended for the MRSA identification and *mecA*/*mecC* detection. Additionally, two laboratories that registered, still had technical issues and were not able to set up the methods for this trial in time and therefore did not upload any data.

It is recommended to use the EURL-AR recommended PCR method to improve the results, to be able to correctly identify the *S. aureus* strains and detect both *mec*-gene variants. Further laboratories showing deviations had issues with species identification and should therefore look deeper into their methods and use the recommended EURL-AR method as well as check the management and QC in the laboratory to avoid possible switches or contaminations of test strains.

4.2 *spa* typing

For *spa* typing, 17 laboratories participated in the trial which indicates a good level of participation even though no MRSA monitoring is ongoing in the EU. The results uploaded were excellent, with sixteen of the 17 laboratories showing no deviation. The deviating *spa*-type result was most likely caused by handling of the test strain as the obtained *spa* type could be from another EQAS test strain (MSSA).

Results from MSSA and non-*S. aureus* were not required to be *spa* typed by the protocol, however this might have not been very clearly stated as many laboratories have uploaded *spa* types for the MSSA strains instead of selecting the “non applicable” option. This is rather controversial as it is possible to *spa* type MSSA strains and the results would have been very good in general if that was the case, however, as was mentioned in the protocol that this was not intended, these results were not taken up in the evaluation. This can be explained by the fact that, in case of MRSA monitoring, the laboratories would not be required to provide *spa* types for the detected MSSA, but only the MRSA. To avoid unfair deviations resulting from uploading *spa* types instead of the “non applicable” option, none of the results uploaded for these strains were evaluated in this trial.

5. Conclusion

In general, the results of the fifth MRSA EQAS 2013 demonstrate that most participating laboratories have set up the relevant methods and are able to identify MRSA in a reliable fashion using molecular methods. As there is currently no MRSA monitoring in the EU, it is not the NRL's first priority to have this method running, however, given the importance of MRSA, the EURL-AR will strive towards having the remaining laboratories setting up the MRSA identification and confirmation at the MS level. The frequency of the MRSA EQAS will be reduced to every second year to keep preparedness at country level for MRSA detection and identification.

The participation in the *spa* typing module indicates that 53% of the laboratories have this method available. However, still fifteen laboratories participating in the MRSA EQAS 2013 did not take part in the optional *spa* typing module. This might indicate that these laboratories have prioritized *spa* typing lower or did not set up this methodology.

The results of the *spa*-typing module show that *spa* typing as expected is a reproducible method which did not cause major problems relative to the execution or interpretation in the participating laboratories. Only one deviation was observed in this module due to incorrect *spa* type assignment which might be due to switching or cross contamination between two EQAS strains, which again shows the need of controlling the analysis process in every step, to obtain a reliable final result.

6. References

European Commission, 2008. COMMISSION DECISION of 20 December 2007 concerning a financial contribution from the Community towards a survey on the prevalence of *Salmonella* spp. and Methicillin-resistant *Staphylococcus aureus* in herds of breeding pigs to be carried out in the Member States. Decision 2008/55/EC in: http://ec.europa.eu/food/food/biosafety/salmonella/impl_reg_en.htm

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Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J Clin Microbiol. 1999 Nov;37(11):3556-63.

Stegger M, Andersen PS, Kearns A, Pichon B, Holmes MA, Edwards G, Laurent F, Teale C, Skov R, Larsen AR. 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.

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M00-06-001/01.12.2011

Appendix 1- EURL-AR EQAS pre-notification

EQAS 2013 FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING OF *E. COLI*, STAPHYLOCOCCI AND ENTEROCOCCI, AND IDENTIFICATION AND TYPING OF MRSA

The EURL-AR announces the launch of another EQAS, thus providing the opportunity for proficiency testing which is considered an essential tool for the generation of reliable laboratory results of consistently good quality.

This EQAS consists of antimicrobial susceptibility testing of eight *E. coli* isolates, eight staphylococci and eight enterococci isolates. The EQAS on identification and typing of MRSA strains will consist on the confirmation of identification and methicillin resistance by multiplex PCR for the eight Staphylococcus isolates and two isolates provided additionally for the MRSA trial. Additionally, *spa* typing of the strains confirmed as MRSA is offered as an optional module.

Additionally, quality control (QC) strains *E. coli* ATCC 25922 (CCM 3954), *E. faecalis* ATCC 29212 (CCM 4224), *S. aureus* ATCC 25923 (CCM 3953) (for disk diffusion) and *S. aureus* ATCC 29213 (CCM 4223) (for MIC) will be distributed to new participants.

This EQAS is specifically for NRL's on antimicrobial resistance and additional designated laboratories performing the selective isolation and identification of MRSA from pig farms. These laboratories do not need to sign up to participate but are automatically regarded as participants. You may contact the EQAS-coordinator, if you wish to inform of changes. Participation is free of charge for all above-mentioned designated laboratories.

TO AVOID DELAY IN SHIPPING THE ISOLATES TO YOUR LABORATORY

The content of the parcel is "UN3373, Biological Substance Category B": eight *E. coli*, ten staphylococci, eight enterococci and for new participants also the QC strains mentioned above. Please provide the EQAS coordinator with documents or other information that can simplify customs procedures (e.g. specific text that should be written on the pro-forma invoice). To avoid delays, we kindly ask you to send this information already at this stage.

TIMELINE FOR RESULTS TO BE RETURNED TO THE NATIONAL FOOD INSTITUTE

Shipment of isolates and protocol: The isolates will be shipped in June 2013. The protocol for this proficiency test will be available for download from the website (www.eurl-ar.eu).

Submission of results: Results must be submitted to the National Food Institute no later than the 6th of September 2013 via the password-protected website. Upon reaching the deadline, each participating laboratory is kindly asked to enter the password-protected website once again to download an automatically generated evaluation report.

EQAS report: A report summarising and comparing results from all participants will be issued. In the report, laboratories will be presented coded, which ensures full anonymity. The EURL-AR and the EU Commission, only, will have access to un-coded results. The report will be publicly available.

Next EQAS: The next EURL-AR EQAS that we will have is on antimicrobial susceptibility testing of *Salmonella* and *Campylobacter* which will be carried out in October 2013

Please contact me if you have comments or questions regarding the EQAS.
Sincerely,

Lina Cavaco - EURL-AR

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Appendix 2- Participant List

Registered	MRSA ID	spa	Institute	Country
x	x	x	Austrian Agency for Health and Food Safety	Austria
x	x	x	Veterinary and Agrochemical Research Centre	Belgium
x			Nacional Diagnostic and Research Veterinary Institute	Bulgaria
x	x		Croatian Veterinary Institut	Croatia
x	x		Veterinary Services	Cyprus
x	x		SVI Olomouc	Czech Republic
x	x	x	National Food Institute	Denmark
x	x		Estonian Veterinary and Food Laboratory	Estonia
x	x	x	Finnish Food Safety Authority EVIRA	Finland
x	x	x	Agence nationale de sécurité sanitaire ANSES - Ploufragan and Maisons-Alfort	France
x	x	x	Federal Institute for Risk Assessment	Germany
x	x		Veterinary Laboratory of Chalkis	Greece
x	x	x	Central Agricultural Office Veterinary Diagnostic Directorate	Hungary
x	x		Central Veterinary Research Laboratory	Ireland
x	x	x	Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana	Italy
x	x	x	Institute of Food Safety, Animal Health and Enviroment "BIOR"	Latvia
x	x		National Food and Veterinary Risk Assessment Institute	Lithuania
x			Public Health Laboratory	Malta
x	x		Food and Consumer Product Safety Authority (VWA)	Netherlands
x	x		Central Veterinary Institute of Wageningen UR	Netherlands
x	x	x	Veterinærinstituttet	Norway
x	x		National Veterinary Research Institute	Poland
x	x		Laboratorio National de Investigacáo Veterinaria	Portugal
x	x	x	Faculdade de Medicina Veterinária UTL	Portugal
x	x		Institute for Hygiene and Veterinary Public Health	Romania
x	x		Institute for Diagnosis and Animal Health	Romania
x	x		State Veterinary and Food Institute (SVFI)	Slovakia
x	x	x	National Veterinary Institute	Slovenia
x	x		Laboratorio Central de Sanidad, Animal de Santa Fe	Spain
x	x	x	VISAVET Health Surveillance Center, Complutense University	Spain
x	x	x	National Veterinary Institute, SVA	Sweden
x	x	x	Vetsuisse faculty Bern, Institute of veterinary bacteriology	Switzerland
x	x	x	Faculty of Veterinary Medicine,	The Netherlands
x	x	x	The Veterinary Laboratory Agency	United Kingdom

Appendix 3- Expected results

CODE	species	*spa*	*mecA*	*mecC*	status	MRSA conclusion	*Spa* MSSA
EURL ST 7.1	*S. aureus*	t843	neg	pos	MRSA	Positive	
EURL ST 7.2	*S. aureus*	Not applicable	neg	neg	MSSA	Negative	(t127)
EURL ST 7.3	*S. aureus*	t1250	pos	neg	MRSA	Positive	
EURL ST 7.4	*S. aureus*	t075	pos	neg	MRSA	Positive	
EURL ST 7.5	*S. aureus*	t2582	pos	neg	MRSA	Positive	
EURL ST 7.6	*S. aureus*	Not applicable	neg	neg	MSSA	Negative	(t3855)
EURL ST 7.7	*S. aureus*	t4571	pos	neg	MRSA	Positive	
EURL ST 7.8	*S. aureus*	t108	pos	neg	MRSA	Positive	
EURL ST 7.9	*S. haemolyticus*	Not applicable	neg	neg	CNS	Negative	
EURL ST 7.10	*S. pseudintermedius*	Not applicable	pos	neg	MRSP	Negative	

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External Quality Assurance System (EQAS) 2013

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Appendix 4- PROTOCOL

For antimicrobial susceptibility testing of *Escherichia coli*, enterococci and staphylococci, and identification and typing of MRSA

1. INTRODUCTION

The organisation and implementation of an External Quality Assurance System (EQAS) on antimicrobial susceptibility testing (AST) of *E. coli*, enterococci and staphylococci, and identification and typing of MRSA is among the tasks of the EU Reference Laboratory for Antimicrobial Resistance (EURL-AR). The EC/Ent/Staph EQAS 2013 will include AST of eight *E. coli*, eight enterococci and eight staphylococci strains and AST of reference strains *E. coli* ATCC 25922 (CCM 3954), *E. faecalis* ATCC 29212 (CCM 4224), *S. aureus* ATCC 25923 (CCM 3953) (for disk diffusion) and *S. aureus* ATCC 29213 (CCM 4223) (for MIC). The above-mentioned reference strains are included in the parcel only for new participants of the EQAS who did not receive them previously. The reference strains are original CERTIFIED cultures provided free of charge, and should be used for future internal quality control for antimicrobial susceptibility testing in your laboratory. The reference strains will not be included in the years to come. Therefore, please take proper care of these strains. Handle and maintain them as suggested in the manual 'Subculture and Maintenance of QC Strains' available on the EURL-AR website (see www.eurl-ar.eu).

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The strains included in the identification and typing of MRSA are the eight staphylococci strains for AST together with two additional *Staphylococcus* strains. This component of the EQAS functions as a continuation of the previous MRSA EQAS to evaluate the proficiency of the laboratories on procedures for confirmatory testing and *spa* typing.

Various aspects of the proficiency test scheme may from time to time be subcontracted. When subcontracting occurs it is placed with a competent subcontractor and the National Food Institute is responsible to the scheme participants for the subcontractor's work.

1. OBJECTIVES

This EQAS aims to support laboratories to assess and, if necessary, to improve the quality of results obtained by AST of pathogens of food- and animal-origin, with special regard to *E. coli*, enterococci and staphylococci. Further objectives are to evaluate and improve the comparability of surveillance data on antimicrobial susceptibility of *E. coli*, enterococci and staphylococci reported to EFSA by different laboratories, and to harmonise the breakpoints for antimicrobial susceptibility used within the EU. Additionally, with the MRSA confirmation and *spa* typing components included in this iteration, we intend to continue the harmonization and /or implementation process of MRSA monitoring at the NRL level.

2. OUTLINE OF THE EC/ENT/STAPH EQAS 2013

2.1. Shipping, receipt and storage of strains

In June 2013, the National Reference Laboratories for Antimicrobial Resistance (NRL-AR) will receive a parcel containing eight *E. coli*, eight enterococci and ten staphylococci strains from the National Food Institute, Denmark (two of the staphylococci strains are to be included in the MRSA components, only). This parcel will also contain reference strains, but only for participants who did not receive them previously. All strains belong to UN3373, Biological substance, category B. Extended spectrum beta-lactamase (ESBL)-producing strains as well as methicillin resistant *Staphylococcus aureus* (MRSA) will be included in the selected material.

The reference strains are shipped lyophilised, while the test strains are stab cultures. On arrival, the stab cultures must be subcultured, and all cultures should be kept refrigerated until testing. A suggested procedure for reconstitution of the lyophilised reference strains is presented below.

2.2. Suggested procedure for reconstitution of the lyophilised reference strains

Please refer to the document 'Instructions for opening and reviving lyophilised cultures' reported on the EURL-AR-website (see www.eurl-ar.eu).

2.3. Antimicrobial susceptibility testing

The strains should be tested for susceptibility to the antimicrobials listed in Tables 1, 2 and 3, using the method implemented in your laboratory for performing monitoring for EFSA and applying the interpretative criteria listed below.

Participants performing minimum inhibitory concentration (MIC) determination should use the values listed in Tables 1, 2 and 3 for interpretation of results. These values represent the epidemiological cut-off values developed by EUCAST (www.eucast.org), and allow categorisation of bacterial isolates into two categories: Resistant or susceptible. A categorisation as intermediate is not accepted, and **intermediate results should be interpreted as susceptible.**

Participants using disk diffusion are recommended to interpret the results according to the breakpoints used routinely. However, when testing *E. coli* by disk diffusion, the interpretation of ciprofloxacin results should be done according to the guidelines described by Cavaco and Aarestrup in J Clin Microbiol. 2009 Sep;47(9):2751-8. Strains must be categorised resistant and susceptible. Also in this case, a categorization as intermediate is not accepted, and **intermediate results should be interpreted as susceptible**.

E. coli

Table 1: Antimicrobials recommended for AST of *Escherichia coli* and interpretative criteria

Antimicrobials for *E. coli*	MIC (µg/mL) **R is >**
Ampicillin, AMP	8
Cefepime	0.125
Cefotaxime, CTX	0.25
Cefoxitin, FOX	8
Ceftazidime, CAZ	0.5
Chloramphenicol, CHL	16
Ciprofloxacin, CIP	0.06
Colistin	2
Florfenicol, FFN	16
Gentamicin, GEN	2
Meropenem, MER	0.125
Nalidixic acid, NAL	16
Sulfonamides, SMX	64
Tetracycline, TET	8
Trimethoprim, TMP	2

Plasmid-mediated quinolone resistance

When performing antimicrobial susceptibility testing of *E. coli*, the interpretative criteria listed in Table 1 for results obtained by MIC-determination detect plasmid mediated quinolone resistant test strains. When interpreting a disk diffusion result, reference should be made to the guidelines in described by Cavaco and Aarestrup in J Clin Microbiol. 2009 Sep;47(9):2751-8.

Beta-lactam resistance

Confirmatory tests for ESBL production are mandatory on all strains resistant to cefotaxime (CTX), ceftazidime (CAZ) or meropenem.

Confirmatory test for ESBL production requires use of both cefotaxime (CTX) and ceftazidime (CAZ) alone and in combination with a β-lactamase inhibitor (clavulanic acid). Synergy is defined either as i) a ≥ 3 twofold concentration decrease in an MIC for either antimicrobial agent tested in combination with clavulanic acid vs. its MIC when tested alone (E-test 3 dilution steps difference; MIC CTX : CTX/CL or CAZ : CAZ/CL ratio ≥ 8) or ii) a ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid vs. its zone when tested alone (CLSI M100 Table 2A; Enterobacteriaceae). The presence of synergy indicates ESBL production. Resistance to cefepime gives further indication of ESBL production.

Confirmatory test for carbapenemase production requires the testing of meropenem (MER).

Detection of AmpC-type beta-lactamases can be performed by testing the bacterium for susceptibility to cefoxitin (FOX). Resistance to FOX could indicate the presence of an AmpC-type beta-lactamase, that may be verified by PCR and sequencing.

The classification of the phenotypic results should be based on the most recent EFSA recommendations (EFSA 2012), indicating as:

- Presumptive ESBL: strains with positive synergy test, susceptible to cefoxitin and resistant to cefepime
- Presumptive ESBL+pAmpC: -strains with positive or negative synergy test, resistant to cefoxitin and resistant to cefepime
- Presumptive pAmpC phenotype: -strains with negative synergy test
- Presumptive carbapenemase phenotype: -strain resistant to meropenem
- Unusual phenotype: any other combinations

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The EURL-AR aims to harmonise with the new EU monitoring and the EUCAST expert rules. Accordingly, MIC values and relative interpretation of cefotaxime, ceftazidime and meropenem used for detection of beta-lactamase-producing strains in this EQAS should be reported as found.

Enterococci

Table 2: Antimicrobials recommended for AST of *Enterococcus* spp. and interpretative criteria

Antimicrobials for enterococci	MIC (µg/mL) **R is >** *E. faecium*	MIC (µg/mL) **R is >** *E. faecalis*
Ampicillin, AMP	4	4
Chloramphenicol, CHL	32	32
Ciprofloxacin, CIP	4	4
Daptomycin, DAP	4	4
Erythromycin, ERY	4	4
Gentamicin, GEN	32	32
Linezolid, LZD	4	4
Quinupristin-dalfopristin (Synercid), SYN	4*	Not applicable
Teicoplanin	2	2
Tetracycline, TET	4	4
Tigecycline, TGC	0,25	Not applicable
Vancomycin, VAN	4	4

*DANMAP 2009 (www.danmap.org)

Identification of the *Enterococcus* spp.

In 2013, species identification of the Enterococci must be performed by the NRLs using in-house methods or adopting the protocol available on the EURL-AR website under: http://eurl-ar.eu/233-protocols.htm.

Staphylococci

Eight of the staphylococci strains sent should be tested both in the AST component and in the MRSA components (EURL ST-7.1 to ST-7.8) whereas the two additional strains (EURL ST-7.9 and 7.10) are intended for the MRSA EQAS components only.

Table 3: Antimicrobials recommended for AST of *Staphylococcus aureus* and interpretative criteria

Antimicrobials for *S. aureus*	MIC (µg/mL) **R is >**
Cefoxitin, FOX	4
Chloramphenicol, CHL	16
Ciprofloxacin, CIP	1
Clindamycin, CLI	0.25
Erythromycin, ERY	1
Florfenicol, FFN	8
Gentamicin, GEN	2
Linezolid, LNZ	4
Mupirocin, MUP	1
Penicillin, PEN	0.125*
Quinupristin-dalfopristin (Synercid), SYN	1
Sulfonamides, SMX	128
Tetracycline, TET	1
Trimethoprim, TMP	2
Vancomycin	2

*CLSI M100 Table 2C

Identification and typing of *MRSA*

All ten staphylococci (ST 7.1 to 7.10) are to be used in the MRSA identification and typing components of the proficiency test. Some test strains may be methicillin-resistant. **Confirmation of *mec*A and/or *mecC* presence is mandatory** in this EQAS. For this purpose, you are welcome to use the method you prefer, and upload the result as 'positive' or 'negative'. According to CLSI recommendations (M100, Table 2C), all MRSA should be regarded as resistant to all β-lactam antibiotics.

The ten staphylococci strains are either positive or negative for methicillin resistance and or, represent other methicillin susceptible or resistant *Staphylococcus* species.

As part of the identification and typing of MRSA, handle the strains as follows:

1. Take up the strains from the agar sticks and plate onto a blood agar plate (you may use additional selective media, but it is not compulsory).
2. Incubate 24-48 h at 37 °C.
3. Observe the colony morphology of the isolates on the blood agar plate (colour, appearance, haemolysis). Check for purity.

4. At this stage, the isolates should either be processed immediately or stored under appropriate conditions (–80°C) for later identification and characterisation.

Identification of MRSA

Presumptive MRSA isolates should be confirmed as *Staphylococcus aureus* isolates carrying the *mecA* gene or the *mecC* gene (previously known by $mecA_{LGA251}$) by PCR. There is no need to perform other screening methods (such as screening with either oxacillin or cefoxitin), thus, the presence of the *mecA* or *mecC* gene can be directly confirmed by PCR amplification. The species identification is simultaneously confirmed by using the EURL-AR recommended multiplex PCR protocol (http://eurl-ar.eu/233-protocols.htm) including the amplification of the *spa* gene (specific for *Staphylococcus aureus* species and may be sequenced for *spa* typing), the *mecA*-gene and the *mecC* gene (both encoding methicillin resistance) and the *pvl* gene (encoding the Panton Valentine Leukocidin).

***Spa* typing**

Spa typing of the MRSA isolates may be performed additionally if the laboratory has the capacity to perform and analyse the *spa*-typing data. In case you decide to include *spa* types in the submitted data, these will be evaluated on the accuracy of the *spa* typing.

3. REPORTING OF RESULTS AND EVALUATION

3.1. AST of E. coli, enterococci and staphylococci

Please write your results in the test forms, and enter your results into the interactive web database. In addition, we kindly ask you to report in the database the tested MIC range and/or antimicrobial disk content. Finally, if **you did not use the cut-off values recommended in the protocol for interpretation of AST results, please report the breakpoints used in the database.**

3.2. MRSA identification and typing

Fill in your results in the enclosed MRSA EQAS test forms. Please enter your results into the interactive web database. Please read the detailed description below before entering the web database. When you enter the results via the web, you will be guided through all steps on the screen and you will immediately be able to view and print a submission report of your results.

3.3. General recommendations for data upload

We recommend reading carefully the description reported in paragraph 5 before entering your results in the web database. **Results must be submitted no later than *September, 6th 2013*.** After the deadline when all participants have uploaded results, you will be able to login to the database

once again, and to view and print an automatically generated report evaluating your results. Results in agreement with the expected interpretation are categorised as 'correct', while results deviating from the expected interpretation are categorised as 'incorrect'.

If you experience difficulties in entering your results, please return the completed test forms by e-mail, fax or mail to the National Food Institute, Denmark.

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 us:

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4. HOW TO ENTER RESULTS IN THE INTERACTIVE DATABASE

Please read carefully this paragraph before entering the web page.
Remember that you need by your side the completed test forms and the breakpoint values you used.

Enter the EURL-AR EQAS 2013 start web page (http://thor.dfvf.dk/crl), write your username and password in lower-cases and press enter. Your username and password are the same used in the previous EQAS's arranged by The National Food Institute, Denmark. Do not hesitate to contact us if you experience problems with the login.

You can browse back and forth by using the back and forward keys and by clicking on the EURL logo.

4.1. AST of E. coli, enterococci and staphylococci

Click on either "*E. coli* test results", "enterococci test results" or "staphylococci test results" based on the results you are going to upload. The description reported below is based on *Salmonella* test

result entry, but it is the exact same procedure for entering *E. coli*, enterococci and staphylococci test results.

Click on "Start of Data Entry - Methods and Breakpoints for Salm."

In the next page, you can navigate among fields with the Tab-key and the mouse.

Complete the fields related to the method used for antimicrobial susceptibility testing of *Salmonella* and the brand of discs, tablets, MIC trays, etc.

Fill in the fields related to either antimicrobial disk content or tested MIC range. If you used disk diffusion, please upload the breakpoints used for interpretation of results.

Click on "save and go to next page"

In the data entry pages, enter the obtained values and the interpretation (R, resistant or S, susceptible) for each *E. coli*, enterococcus and staphylococcus strain.

For *E. coli* strains, remember to report also the results for the ESBL detection tests.
For *S. aureus* strains, remember to report also the results for presence/absence of methicillin resistance.

If you did not test for susceptibility to a given antimicrobial, please leave the field empty.

Click on "save and go to next page"

When uploading data on the reference strains, please enter the zone diameters in mm and MIC values in µg/ml. Remember to use the operator keys to show symbols like "equal to", etc... If you do not use CLSI guidelines for AST of the reference strains, please add a comment on the method used.

Click on "save and go to next page"

This page is a menu that allows you to review the input pages and approve your input.

Browse through the pages and make corrections if necessary. Remember to save a page if you make corrections. If you save a page without changes, you will see an error screen. In this case, click on "back" to get back to the page and "go to next page" to continue.

Please complete the evaluation form.

Before approving your input, please be sure that you have filled in all the relevant fields because YOU CAN ONLY APPROVE ONCE! The approval blocks your data entry in the interactive database.

4.2. EQAS on identification and typing of MRSA

Click on "MRSA tests" to start entering your data regarding the MRSA EQAS.

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Please read carefully the instructions on the webpage and start by answering to the questionnaire on the work performed in your laboratory relative to MRSA by clicking on "General MRSA questionnaire".

Please choose the options that more correctly describe your work on MRSA and before you leave this page, click on "Save page" which will take you back to the previous menu. Then, fill in the methods used in a second page by clicking on "Methods for MRSA test samples."

In the next page you navigate to fields with the Tab-key and mouse.

Fill in what kind of method you have used for the confirmation of MRSA in this EQAS.

Click on "Save and go to the next page"

In the data entry pages for each strain, you enter the obtained results for each of the MRSA EQAS strains (EURL ST 7.1...7.10).

If you wish to participate in the *spa* typing trial, you will have the option to include the *spa*-typing results.

If you have performed the *spa* typing, choose the *spa* type from the list. If you did not perform *spa* typing, leave the field blank. In case the isolate is not a methicillin resistant *Staphylococcus aureus*, choose "not applicable (N/A)". Click on "save and go to next page" to navigate to the next sample results, until you finish uploading all your data.

From the last result sheet you get into the general menu, from where you can review the input pages: Browse through the pages and make corrections if necessary. Remember to save a page if you make any corrections. If you save a page without changes, you will see an error screen, and you just have to click on "back" to get back to the page and "go to next page" to continue.

At the end, approve your input. Be sure that you have filled in all the results before approval, as YOU CAN ONLY APPROVE ONCE! The approval blocks your data entry in the interactive database, but allows you to see and print the submitted results.

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Appendix 5- Examples of TEST FORMS - identification and typing of MRSA

Name:

Name of laboratory:

Name of institute:

City:

Country:

E-mail:

Fax:

How many samples did you process in 2012 for MRSA detection? (Choose only one option)

- ☐ less than 50
- ☐ 50-100
- ☐ 101- 200
- ☐ 201-400
- ☐ more than 400

Which kind of samples did you process in your laboratory for MRSA detection in 2012?

- ☐ dust swabs
- ☐ nasal swabs
- ☐ skin swabs
- ☐ faecal samples
- ☐ Boot swabs
- ☐ other matrices Specify:

Which was the origin of the samples processed for MRSA detection in 2012?

- ☐ from humans
- ☐ environmental
- ☐ from animals if you check this answer, please answer the next question
- ☐ other . Specify:

The samples processed from animals included samples obtained from which species:

- ☐ pigs
- ☐ cattle
- ☐ poultry
- ☐ pets
- ☐ other . Specify:

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Any other Comments:

TEST FORM – MRSA methods

Method used for confirmatory testing of MRSA (choose only one option)

- ☐ PCR using the new EURL recommended multiplex PCR protocol (*spa, mecA, mecC,*and *pvl*)
- ☐ PCR using the old EURL multiplex PCR protocol (16S rDNA, *mecA* and *nuc*)
- ☐ modified protocol but same multiplex PCR primers
- ☐ other published PCR method
- ☐ in- house PCR method

Describe if you did not use the new EURL recommended method and justify your choice**:**

Method used for confirmation of species identification (choose only one option)

- ☐ PCR using the new EURL recommended multiplex PCR protocol (*spa, mecA, mecC,*and *pvl*)
- ☐ PCR using the old EURL multiplex PCR protocol (16S rDNA, *mecA* and *nuc*)
- ☐ biochemical methods
- ☐ other published PCR method
- ☐ in-house PCR method

Describe if you did not use the EURL recommended method and justify your choice**:**

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TEST FORM
Entry data Sample EURL-ST 7.1

Date the testing was started:

Results of confirmatory PCR and species identification: (choose only one option)
☐ no isolates tested, sample negative
☐ *mecA* positive, but coagulase-negative Staphylococci (MRCNS)
☐ *mecA* positive, but *S . pseudintermedius* (MRSP)
☐ *mecA and mecC* negative and not *S . aureus* (CNS)
☐ *mecA and mecC* negative, *S. aureus* (MSSA)
☐ *mecA* positive, *S. aureus* (MRSA)
☐ *mecC* positive, *S. aureus* (MRSA)

***Spa* Typing (optional): *spa* type** (choose only one option)
☐ Not performed
☐ Not applicable (N/A)
☐ t011
☐ t021
☐ t034
☐ t075
☐ t108
☐ t127
☐ t337
☐ t524
☐ t571
☐ t843
☐ t899
☐ t1250
☐ t1333
☐ t1430
☐ t1730
☐ t1793
☐ t2510
☐ t2582
☐ t2922
☐ t3855
☐ t4571
☐ t4795

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