

# Report of the 1st External Quality Assurance System on Isolation, Identification and Typing of Methicillin resistant *Staphylococcus aureus* (MRSA) from Dust Samples, year 2009



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## **Community Reference Laboratory – Antimicrobial Resistance**

**REPORT OF THE 1<sup>ST</sup> EXTERNAL QUALITY ASSURANCE SYSTEM ON ISOLATION, IDENTIFICATION AND TYPING OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) FROM DUST SAMPLES, YEAR 2009**

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## Index

<b>1. INTRODUCTION</b> .....	<b>3</b>
<b>2. MATERIALS AND METHODS</b> .....	<b>4</b>
2.1 PARTICIPANTS .....	4
2.2 SAMPLES .....	4
2.3 ISOLATION AND IDENTIFICATION OF MRSA FROM DUST SAMPLES .....	6
2.4 TYPING .....	6
2.5 DISTRIBUTION .....	6
2.6 PROCEDURE .....	6
<b>3. RESULTS</b> .....	<b>7</b>
3.1 METHODS USED BY EQAS-PARTICIPANTS .....	8
3.2 MRSA ISOLATION AND IDENTIFICATION.....	9
3.2.1 Results per laboratory .....	10
3.2.2 Results per sample .....	11
3.3 SPA TYPING OF THE MRSA STRAINS .....	12
3.3.1 Results per laboratory .....	12
3.3.2 Results per sample .....	13
<b>4. DISCUSSION</b> .....	<b>15</b>
4.1 MRSA ISOLATION AND IDENTIFICATION.....	15
4.2 SPA TYPING .....	15
<b>5. CONCLUSION</b> .....	<b>16</b>
<b>6. REFERENCES</b> .....	<b>16</b>
<b>APPENDIX 1 CRL-AR MRSA EQAS 2009 PRE-NOTIFICATION</b> .....	<b>17</b>
<b>APPENDIX 2- PARTICIPANT LIST</b> .....	<b>19</b>
<b>APPENDIX 3 EXPECTED RESULTS</b> .....	<b>20</b>
<b>APPENDIX 4- PROTOCOL FOR THE MRSA EQAS</b> .....	<b>21</b>
<b>APPENDIX 5 - TEST FORMS MRSA EQAS 2009</b> .....	<b>28</b>



## 1. Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) have recently emerged in food producing animals. The emergence of this potential zoonotic pathogen in animals has raised the concerns of potential transmission to humans from the animal reservoirs and consequences on Public Health. In 2008, the European Commission has taken a Decision of screening the population of pig farms in Europe for this new emerging pathogen, taking this screening in 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 Community Reference Laboratory for Antimicrobial Resistance (CRL-AR) has taken part in the development and support of the laboratories in this task by providing assistance in setting up the methods for isolation, identification and typing of methicillin resistant *Staphylococcus aureus*, and providing training in the specific methods on individual basis and on a MRSA training course that was organized in April 2009.

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

This was the first EQAS performed on MRSA isolation, identification and typing. It was based on dust samples, rather than isolates and included isolation steps besides the identification and also a molecular biology component in the detection of methicillin resistance. The detection of the *mecA* gene was included as a mandatory part for confirmation of MRSA. An optional sequence based typing module based on *spa* typing was also offered. The *spa* typing trial aimed at increasing the capacity of laboratories by performing a standard typing method which can be easily performed, and which results are comparable and useful to assess the epidemiology of MRSA.

At the starting point of this EQAS, the baseline study had already been performed. Therefore the laboratories should have set up the methods for its performance. In this view, and the EQAS results may



also be used to assess, although retrospectively, the quality of the performances of the laboratories on the Baseline survey, and the quality of data provided to EFSA.

As the first EQAS of this kind, no thresholds have been set in advance to evaluate the performance of the participating laboratories, nor classify the results of this EQAS. This discussion will be taken at the annual workshop, if further MRSA EQAS are performed within the CRL-AR activities in the future.

The MRSA EQAS was organized by the National Food Institute (DTU Food), Copenhagen, Denmark and the verification/confirmation of the strains used in the preparation of the test samples was performed at the Statens Serum Institute in Copenhagen, Denmark.

The Individual laboratory data is kept confidential and is only known to the participating laboratory, the EQAS Organizer (DTU Food) and the European Commission. All summary conclusions are made public.

## **2. Materials and Methods**

### **2.1 Participants**

A pre-notification announcement of the MRSA EQAS 2009 was issued by e-mail to our CRL-AR network on March 20, 2009 and National Reference laboratories (NRL) that have participated in the baseline studies on MRSA were invited to participate. In the countries, where a different laboratory participated in the baseline study, the NRL's were notified to provide the CRL-AR with the contact information of the participating laboratory in the respective countries. As soon as the CRL-AR received the contact information of the additional laboratories that had participated in the baseline studies, the laboratories were notified by the MRSA EQAS coordinator using the same pre-notification announcement.

Participation was free of charge but each laboratory was expected to cover expenses associated with their own analysis.

### **2.2 Samples**

Eight dust samples were prepared and dispatched for isolation of MRSA, identification, and typing of the obtained isolates. The samples were artificially prepared, to contain either methicillin-resistant, or



methicillin-susceptible *Staphylococcus aureus* and/or coagulase negative staphylococci strains, besides the normal dust flora that would be present in dust collected in the environment of a pig farm.

### **2.2.1 Preparation of samples:**

The dust used in the preparation of the samples was collected from the pig facilities at the National Veterinary Institute in Copenhagen, Denmark, and was tested previously MRSA-free, using the standard method recommended in the baseline studies. Furthermore, the dust collected from these facilities had been collected repeatedly and tested periodically for the last 6 months and was consistently found MRSA-negative.

The MRSA isolates chosen for the MRSA EQAS (CRL-MRSA 1.1-1.8) were prepared in advance and subcultured in week 24 and re-subcultured on the day before sample preparation. For the sample standardization, suspensions equal to Mc Farland 0.5 were prepared in saline tubes of the relevant isolates and for each suspension to contain about  $10^8$  cfu/ml. The suspensions were further diluted 1:10, to  $10^7$  cfu/ml of which 100  $\mu$ l were used to inoculate the dust samples containing 0,1g dust. The expected inoculum was about  $10^6$  cfu per sample and was confirmed by performing serial dilution and colony forming unit counts in duplicate from all isolate suspensions.

The strains used for the EQAS were selected from the strain collections at the National Food Institute and the identification was confirmed by PCR and 16S rDNA. Furthermore, the *spa* type of the MRSA isolates was 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 the Statens Serum Institute (SSI), Copenhagen, Denmark. The results obtained after preparation of the contaminated dust samples were confirmed at DTU-Food, by testing (isolation, identification and typing) of three sample sets just after preparation. Stability testing was performed in one set of eight samples per week, during one month, using the protocol for isolation and identification of MRSA recommended, confirming that the results of the isolation/identification of MRSA from the eight EQAS samples were repeatable.



### 2.3 Isolation and identification of MRSA from dust samples

The protocol for isolation and identification of the MRSA isolates contained in the dust samples was made available on the CRL website ([www.crl-ar.eu](http://www.crl-ar.eu)) (App.4).

### 2.4 Typing

The isolates used for the sample preparation were typed by a single locus sequence typing method - *spa* typing (Shopsin *et al.* 1999) and the results were verified by parallel *spa* typing conducted at the SSI. Furthermore, the *spa* typing results were verified a third time in the isolates obtained from prepared samples.

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

### 2.5 Distribution

The samples were prepared just before shipment to avoid problems with stability and were kept at room temperature. The tubes containing the dust samples 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 June 17, 2009 to June 19.

### 2.6 Procedure

The laboratories were instructed to download the protocol and test forms (App. 4 and 5) available in English only, from <http://www.crl-ar.eu>

The EQAS protocol was based on the EC baseline protocol for isolation of MRSA from dust samples, however, with slight changes in the volume of the media needed in the enrichment steps, since the samples were sent as dust samples in a tube and not dust swabs. The method recommended for the identification of the isolates was the CRL multiplex PCR which would allow identification of *Staphylococcus aureus* and also identify the *mecA* gene, mandatory for the classification of isolates as MRSA (App 4).



After completion of the tests, the laboratories were requested to enter the obtained results into an electronic record sheet in the CRL-AR web based database through a secured individual login, or alternatively send the record sheets from the enclosed protocol by fax to DTU Food (App 5). The database was activated June 25, 2009 and closed September 15, 2009.

The samples were categorised as positive or negative in function of the isolation and identification of MRSA isolates and no quantitative variable was introduced. Negative samples could be subdivided into different categories (negative sample, MSSA, MRCNS, 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.

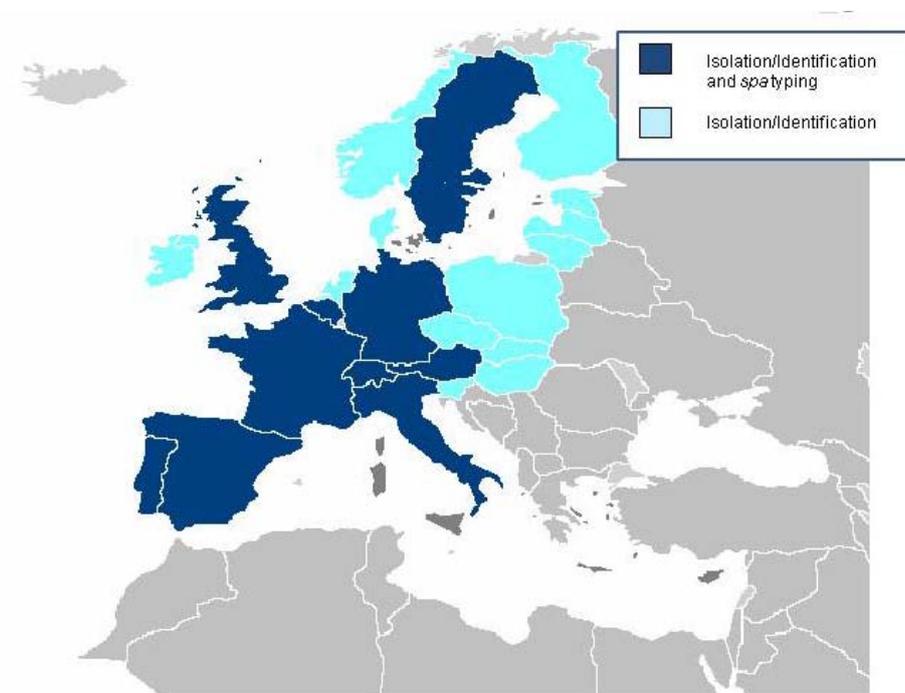
Optionally, results of *spa* types were requested for typing the positive MRSA samples. In this case, the participants would be invited to choose the obtained *spa* type from a pick list, and the typing result would be evaluated against the expected *spa* type and classified into correct or incorrect.

### **3. Results**

A total of 24 laboratories, including 17 NRL and five non-NRLs incl. laboratories from Switzerland and Norway responded to the pre-notification, and were enrolled in the EQAS. Additionally, one laboratory that is not an NRL and did not participate in the baseline studies participated in the EQAS, however, the results obtained at this particular laboratory were excluded from this report and will not be further mentioned.

When the deadline for submitting results was reached, 23 laboratories in 23 European countries (including Switzerland and Norway) had uploaded data (one of the NRLs, Lab # 40 was unable to perform the testing and did not upload data due to technical problems). The following countries provided data to at least one of the EQAS components (also shown below in Figure 1): Austria, Belgium, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Lithuania, the Netherlands, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland and UK.

Figure 1- Participating Countries



Participating countries are marked in light blue for laboratories participating in MRSA isolation and identification and dark blue for those additionally participating in the *spa* typing.

In the description of results, arbitrary thresholds of quality limits have not been used, and deviations have been classified as incorrect results without further classification.

### 3.1 Methods used by EQAS-participants

Of the 23 laboratories submitting results all (100%) of the laboratories participated in the MRSA isolation and identification module whereas only nine (39%) 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.

Most laboratories (n=20) reported that isolation has been performed according to the protocol that was provided by the CRL for this EQAS and any changes in media, concentrations of antibiotics, etc. were noted. The differences reported by some laboratories from the method described in the original protocol



were: use of 5 mg/L oxacillin instead of 3,5 mg/L cefoxitin in the second enrichment step in one laboratory, use of phenol red mannitol broth with 5 mg/l ceftizoxime en 75 g/l aztreonam in another lab and use of different selective plates for the selective isolation, including CHROMagar MRSA (Mast Diagnostica), MRSA Select (Bio-Rad), Oxacillin Resistance Screening Agar (ORSAB) (Oxoid); Chrom ID MRSA Agar (bioMérieux) and Baird Parker with a Penicillin disk, were reported. The species identification of *Staphylococcus aureus* was performed using the CRL recommended PCR in 13 laboratories and the other referred methods for identification including other published PCR and RT-PCR methods and biochemical reactions. The detection of the *mecA* gene was mostly performed by PCR, using the CRL recommended PCR primers (n=12), and using other published PCR methods in the remaining laboratories.

### 3.2 MRSA isolation and identification

A total of 179 tests (one laboratory has reported results for three samples only, whereas the remaining reported results for all the eight samples) have been performed and the overall result indicate that 166 tests were correct, corresponding to 92.7% and a total of 13 deviations were observed (7.3%), including 11 (6.2%) false negative results and 2 (1.1%) false positive results.

**Table 1-. The overall performance of MRSA isolation and identification, 2009.**

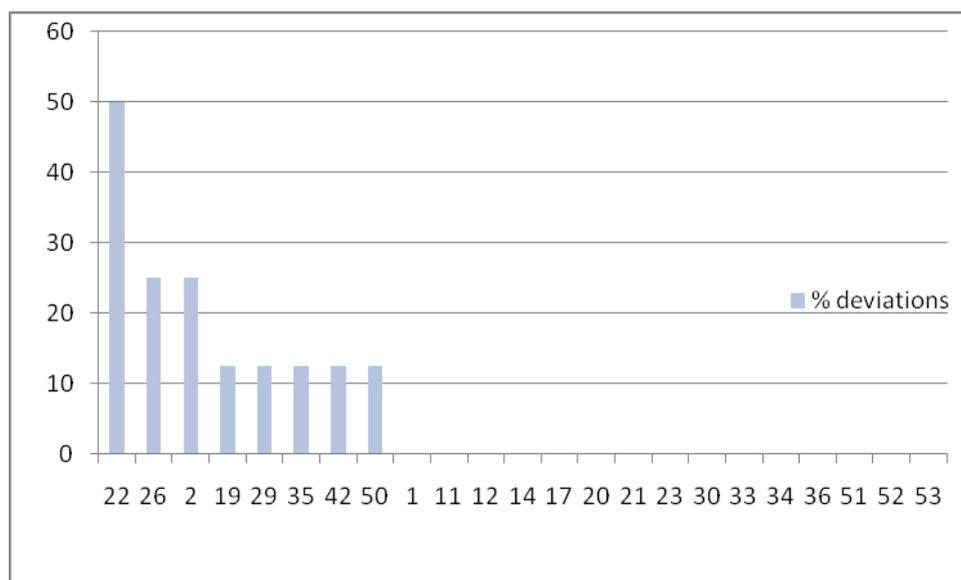
Isolation of MRSA from dust samples		Correctly classified samples	
Number of performed tests		Number of correct tests N(%)	
n	%	N	%
179	100	166	92.7
Number of expected negative tests		Number of correctly identified negative tests	
n	%	N	%
88	49.2	86	97.7
Number of expected positive tests		Number of correctly identified positive tests	
n	%	N	%
91	50.8	80	87.9%



### 3.2.1 Results per laboratory

The individual laboratory results of this first MRSA EQAS on isolation and identification of MRSA from dust samples, in general showed a good performance by most laboratories, since the majority were able to recover and correctly identify the isolates from the dust samples provided. In fact, 15 out of the 23 laboratories were able to correctly assign all the tested samples as either MRSA positive or negative whereas only three laboratories have been able to classify 6 or less samples correctly (Graph 1). One laboratory, Lab #11 has only submitted the final interpretation for three of the positive samples (CRL-MRSA 1.5, 1.6 and 1.8) and has therefore only reported three correct results, although no deviating results were reported.

Graph 1- Deviating results per laboratory. The laboratories were ranked by decreasing percentage of deviation.



As described above, most errors were caused by a lack of sensitivity of the methods used, leading to false negative results. The two false positive results were both observed in the same laboratory (Lab #2) and might have been caused by cross contamination of the samples, since *spa* typing confirmed the same types which were expected in isolates present in two other samples sent out in the MRSA EQAS.



Among the eight laboratories reporting deviating results five laboratories reported one deviation, two laboratories reported two deviations and only one laboratory has reported four deviating results (Lab #22). The latter laboratory (Lab#22) had 50% deviation in the results and these deviations were demonstrating lack of sensitivity of the methods used, since all the samples were considered negative for MRSA and the four expected positive samples were found to contain only coagulase negative staphylococci that were part of the background flora.

### 3.2.2 Results per sample

As described above, most deviations were caused by lack of sensitivity of the method and only two deviations were caused by lack of specificity, therefore, most deviations were observed for the samples expected to contain MRSA isolates. We observed that the MRSA-negative samples (CRL-MRSA- 1.1, 1.2, 1.4 and 1.7) did not cause problems in most laboratories.

Only Lab #2 obtained false-positive deviations by reporting samples CRL-MRSA 1.1 and 1.4 as MRSA-positive. Sample CRL-MRSA 1.1 contained a MSSA strain that was not expected to be isolated when performing the selective isolation procedure, nor misclassified if the *mecA* PCR was performed correctly. However, this sample was identified as MRSA-positive by Lab #2 and the *spa* type obtained by typing the isolate obtained corresponded to the isolate that was expected to be isolated from sample CRL-MRSA 1.8. In the same way, sample 1.4 which contained a methicillin-resistant coagulase negative *Staphylococcus* strain (*S. simulans*) and not a MRSA would not be typable by *spa* typing, which in this case was done obtaining the same *spa* type expected for the strain isolated from sample CRL-MRSA 1.5. Therefore, it is suspected that the deviating false positive results are due to cross contamination which might have occurred during the isolation process and lead to characterization of isolates not expected to be present in the initial sample.

The sample causing most deviations was CRL MRSA 1.3 which caused five deviations. These were related to the failure in detection of this isolate from the dust sample, possibly related to different properties, such as a slight different morphology (smaller and light colonies). This might have led to disregard this strain when selecting suspected isolates for MRSA-identification.

Samples CRL-MRSA 1.5, 1.6 and 1.8 caused three, one and two incorrect results, respectively. There are no obvious explanations for these deviations, besides lack of sensitivity in the isolation/identification methods that might have lead to failure in recovery and detection of these isolates.



Additionally, one laboratory (Lab #17) reported the use of an additional enrichment to increase the recovery of MRSA strains from the EQAS samples by streaking out a second time all selective broth cultures after refrigeration at 4°C during 4 weeks and the use of different selective plates, which they refer has increased their recovery rate.

### **3.3 *spa* typing of the MRSA strains**

The *spa* typing module counted with the participation of 10 laboratories, which have uploaded *spa* typing data (Lab #2, #14, #17, #21, #33, #34, #35, #42, #50, #53). Additionally, lab #1 has only uploaded one result indicating N/A for sample CRL-MRSA 1.1, but did not upload any *spa* typing results for any of the MRSA-positive samples.

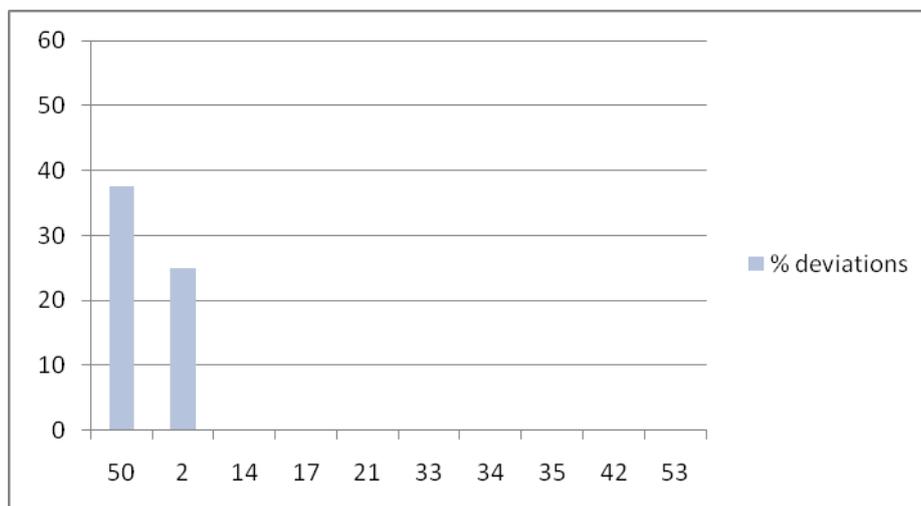
The reported results for *spa* typing included 54 tests and the general results show that 49 (90.7%) of results were considered correct and only five (9.8%) deviations were observed. The observed deviations were due to incorrect *spa* type assignment (n=2), *spa* typing of sample expected to be negative (n=2), or choice of option N/A for a sample that should have rendered an isolate for *spa* typing (n=1).

#### **3.3.1 Results per laboratory**

Most of the laboratories have only reported the results regarding the sample previously found positive for MRSA, whereas no more than three have reported N/A (not applicable) results to the isolates considered MRSA negative, as required. Regarding these results, interdependence between the isolation/identification results has to be considered. The choice of the option N/A would be considered a deviation if the *spa* typing should have been applied to a MRSA positive sample and it would be considered correct if the isolate was not expected to be an MRSA.

By laboratory, the results show that the laboratories uploading *spa* typing data have in general obtained good results with eight laboratories without any deviations and two laboratories (Lab #50 and #2) which have registered respectively three and two deviations out of eight and six samples tested, respectively.

**Graph 2- Deviating results per participating laboratory. The laboratories were ranked by decreasing percentage of deviation.**



### 3.3.2 Results per sample

In this module, samples CRL-MRSA 1.3, 1.5, 1.6, and 1.8 were expected to be identified positive for MRSA and therefore optional *spa* typing was offered for the respective isolates found, whereas the remaining negative samples (CRL-MRSA 1.1, 1.2, 1.4 and 1.7) would not render a positive MRSA isolate for typing and be therefore classified as N/A.

The *spa* type t075 was correctly identified, whenever an isolate from sample CRL-MRSA 1.3 was typed (n=7). However three laboratories that uploaded data for *spa* typing did not found this sample positive, but no deviations were registered since no incorrect *spa* typing result was uploaded.

Regarding sample CRL-MRSA 1.5 and 1.6 and 1.8 one deviation was registered for each sample, whereas the remaining two deviations were caused by reporting of *spa* typing of isolates from samples CRL-MRSA 1.1 and 1.4 that were expected negative for MRSA.

The deviation observed for sample CRL-MRSA 1.5 (Lab #50) was also due to lack of isolation of the MRSA isolate and reporting of N/A, since this sample was expected to contain MRSA type t034. The deviations obtained by the same laboratory for CRL-MRSA 1.6 and 1.8 are due to reporting incorrect *spa* types. It is interesting to note that the reported *spa* types t021 and t108 would be correct if reported



inversely (t108 and t021, respectively), suggesting a switch between the *spa* typing results of the isolates obtained from samples CRL-MRSA 1.6 and 1.8.

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

Sample number	N part labs	expected repeat succession	expected <i>spa</i> type	correct	Deviating results
CRL MRSA 1. 1	5	None – <i>mecA</i> negative should not be isolated	N/A (t011)	4	t021
CRL MRSA 1. 2	3	None- <i>S. haemolyticus</i> , <i>spa</i> negative	N/A	3	-
CRL MRSA 1. 3	7	r11r19r21r21r12r21r17r34r24r34r22r25	t075	7	-
CRL MRSA 1. 4	4	None- <i>S. simulans</i> , <i>spa</i> negative	N/A	3	t034
CRL MRSA 1. 5	10	r08r16r02r25r02r25r34r24r25	t034	9	N/A
CRL MRSA 1. 6	10	r08r16r02r25r24r25	t108	9	t021
CRL MRSA 1. 7	3	None- Negative control	N/A	3	-
CRL MRSA 1. 8	10	r15r12r16r02r16r02r25r17r24	t021	9	t108



## 4. Discussion

### 4.1 MRSA isolation and identification

In the 2009 CRL MRSA EQAS trial, the samples were prepared with strains selected based on recent findings and the quantity inoculated was rather high to facilitate the recovery of the MRSA isolates in the positive samples which reflects in the good overall results.

The 11 deviations caused by lack of sensitivity can be explained due to lack of sensitivity in the media and methods that might require some further attention and corrective actions to improve the recovery of MRSA from dust samples. Regarding the individual positive samples, five out of 11 deviations due to lack of sensitivity were caused by sample 1.3 which was found more difficult due to differences in the morphology (smaller colonies and light colouring of the colonies on selective plates) that might have led to elimination of this isolate at an early stage, although identification and *mecA* gene detection would easily confirm the MRSA status.

The lack of sensitivity in the methods used in the laboratories deserves further attention. The same method and procedures were followed at the CRL in all tests performed during the preparation and verification of the samples and 100% of the strains were recovered from the samples tested. Furthermore, the stability tests that were performed after sending the samples showed that it was possible to recover the inoculated MRSA isolates until after one month of sample preparation. Therefore, it was expected that laboratories would be able to obtain correct results when using the recommended methods. The variation of media and concentrations of antibiotics might have contributed to a difference in the recovery of some of the samples, although most laboratories refer to the use of the recommended isolation procedures.

### 4.2 *spa* typing

For *spa* typing ten laboratories have participated in the trial and the results were very good in general, with eight of the ten laboratories showing no deviation and only two with three and two deviations, respectively. Most participating laboratories have, however, only uploaded the results of the positive strains they have found, whereas only three laboratories have submitted data for all eight samples.



The deviations observed do not demonstrate any problems with the *spa* typing method, but reflect either the lack of sensitivity of the isolation procedure in the cases where no isolate is obtained for typing, or some problems with cross contamination or sample management that might have caused the deviations observed in *spa* types found switched or attributed to expected negative samples.

## 5. Conclusion

In general, the results of the first MRSA trials demonstrate that most participating labs have set up the methods and are able to isolate MRSA from dust samples. However, there are problems with the sensitivity of the methods used in several laboratories, especially in Lab #22 where no positive isolates were obtained. Few cases were observed where the samples were found positive, when expected negative. These findings indicate cross contamination issues that need to be resolved with aseptic manipulation to avoid contamination of other samples, environment or material (pipettes, etc). Furthermore we observed deviations that might be related to switching between samples which can be controlled by correct and sample management strategies and control of procedures of the process to control any sources of deviation in each of the steps involved in the isolation and identification.

The low participation in the *spa* typing module indicate that *spa* typing is not yet a method that is available in many laboratories in Europe, but the results show that *spa* typing is as expected a reproducible method which did not cause problems relative to the execution or interpretation. The deviations observed were either related to lack of isolation of the MRSA expected in the sample, or due to possible switching of samples or cross contamination, 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](http://ec.europa.eu/food/food/biosafety/salmonella/impl_reg_en.htm)

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.



## Appendix 1 CRL-AR MRSA EQAS 2009 pre-notification

### EQAS 2009 FOR DETECTION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN DUST SAMPLES

The CRL are pleased to announce the launch of another EQAS. The EQAS provides the opportunity for proficiency testing, which is considered an important tool for the production of reliable laboratory results of consistently good quality.

This EQAS offers detection, identification and typing of MRSA from eight dust samples which contain normal bacterial flora present in dust of which some will contain MRSA.

**This EQAS is designed specifically to the laboratories involved in the EU baseline studies which performed the selective isolation and identification of MRSA from dust samples originated from pig farms. If your laboratory has not been involved in the processing of these samples, we would kindly ask you to pass on this message to the relevant contact persons in the laboratory involved with the baseline studies and please return to the CRL-AR with their contact information.**

If you do not send any contact information of an alternative laboratory, we will assume your NRL has been involved in the detection of MRSA in the baseline studies and you do not need to sign up for participation. All who receive this pre-notification are automatically regarded as participants. Participation is free of charge for all NRL's.

### TO AVOID DELAY IN SHIPPING THE ISOLATES TO YOUR LABORATORY

Please remember to provide the EQAS coordinator with import permits or other information that can ease the parcel's way through customs (eg. specific text that should be written on the invoice). As means of avoiding passing the deadline we ask you to send us this information already at this stage. For your information, the contents of the parcel are UN3373 "Biological Substance Category B": Eight tubes containing dust samples that might contain MRSA. The parcels are expected to arrive at your laboratory in June 2009.

**TIMELINE FOR RESULTS TO BE RETURNED TO THE NATIONAL FOOD INSTITUTE** Shipment of isolates and protocol: The samples will be shipped in June 2009. The protocol will be provided via our website. This protocol will be based on the protocol used in the baseline studies and therefore we would like to ask you to have the media referred in the baseline protocol and any materials or reagents needed for the selective isolation procedure, the confirmatory PCR method and *spa* typing (optional) readily available for processing the samples which will need to be processed right upon arrival.

Returning of results: Results must be returned to the National Food Institute, by September 1<sup>st</sup>, 2009. When you enter your results via a password-protected website, an evaluation report of your results will be generated immediately.



EQAS report: When the EQAS is concluded, the data will be collected in an overall report in which it is possible to see all participants' results in comparison. In the report the laboratories will be coded, thus ensuring full anonymity; only the National Food Institute and the EU Commission will be given access to un-coded results.

**Any comments regarding the EQAS, please contact me by e-mail ([licav@food.dtu.dk](mailto:licav@food.dtu.dk)) or by fax (+45 7234 6001).**

Sincerely,

Lina Cavaco

**MRSA EQAS-Coordinator**



## Appendix 2- Participant list

reg	MRSA	spa	Institute	Country
x	x	x	Austrian Agency for Health and Food Safety	Austria
x	x	x	Veterinary and Agrochemical Research Centre	Belgium
x			NRL Antimicrobial resistance on food, Nacional Diagnostic and Research Veterinary Institute	Bulgaria
x	x		SVI Olomouc	Czech Republic
x	x		The National Food Institute	Denmark
x	x		Estonian Veterinary and Food Laboratory	Estonia
x	x		Finnish Food Safety Authority EVIRA	Finland
x	x	x	French Food Safety Agency (AFSSA)	France
x	x	x	Federal Institute for Risk Assessment	Germany
x	x		Central Agricultural Office, Veterinary Diagnostical Directorate	Hungary
x	x		Central Veterinary Research Laboratory	Ireland
x	x	x	Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana	Italy
x	x		National Diagnostic Centre of Food and Veterinary Service	Latvia
x	x		National Veterinary Laboratory	Lithuania
x	x		AHS Deventer	Netherlands
x	x		Veterinærinstituttet	Norway
x	x		National Veterinary Research Institute	Poland
x	x	x	Faculdade de Medicina Veterinária- UTL	Portugal
x	x		State Veterinary and Food Institute (SVFI)	Slovakia
x	x		National Veterinary Institute	Slovenia
x	x	x	Complutense University of Madrid	Spain
x	x	x	National Veterinary Institute, SVA	Sweden
x	x	x	Vetsuisse faculty Bern, Institute of veterinary bacteriology	Switzerland
x	x	x	The Veterinary Laboratory Agency	United Kingdom



## Appendix 3 expected results

Sample ID	<i>mecA</i>	<i>spa</i> type	MRSA status sample
CRL MRSA 1.1	negative	t011	negative
CRL MRSA 1.2	positive, ( <i>nuc</i> negative)	N/A	negative
CRL MRSA 1.3	<b>positive</b>	t075	<b>positive</b>
CRL MRSA 1.4	positive, ( <i>nuc</i> neg)	N/A	negative
CRL MRSA 1.5	<b>positive</b>	t034	<b>positive</b>
CRL MRSA 1.6	positive	t108	<b>positive</b>
CRL MRSA 1.7	N/A	N/A	negative
CRL MRSA 1.8	positive	t021	<b>positive</b>



## **Appendix 4- Protocol for the MRSA EQAS**

### **Protocol for MRSA EQAS 2009**

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For selective isolation, detection and typing of methicillin resistant *Staphylococcus aureus* (MRSA) from dust samples.

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<b>1</b>	<b>INTRODUCTION .....</b>	<b>22</b>
<b>2</b>	<b>OBJECTIVES .....</b>	<b>22</b>
<b>3</b>	<b>OUTLINE OF THE MRSA EQAS 2009 .....</b>	<b>22</b>
3.1	SHIPPING, RECEIPT AND STORAGE OF STRAINS .....	22
<b>4</b>	<b>DETECTION AND IDENTIFICATION OF MRSA FROM DUST SAMPLES .....</b>	<b>23</b>
4.1	SELECTIVE ENRICHMENT AND ISOLATION .....	23
4.2	IDENTIFICATION OF MRSA.....	24
4.3	SPA TYPING .....	24
<b>5</b>	<b>REPORTING OF RESULTS AND EVALUATION .....</b>	<b>24</b>
<b>6</b>	<b>HOW TO ENTER RESULTS IN THE INTERACTIVE DATABASE.....</b>	<b>26</b>

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## Introduction

One of the tasks as the EU Community Reference Laboratory for Antimicrobial Resistance is to organise and conduct an External Quality Assurance System (EQAS). Usually the EQAS have been focused on susceptibility testing, however, due to the recent concern about FA-MRSA (farm acquired methicillin resistant *Staphylococcus aureus*) detected in farms in Europe and the recently performed baseline screening performed aiming at the detection of FA-MRSA in pig farms, we have now decided to perform an EQAS to evaluate the proficiency of the laboratories on the selective isolation procedures for detection of MRSA from dust samples, including their confirmatory testing and *spa* typing.

As referred in the pre-notification, this EQAS is designed specifically for the laboratories involved in the EU baseline studies which performed the selective isolation and identification of MRSA from dust samples originated from pig farms and therefore these laboratories should perform the analysis of the samples sent in this EQAS, using the same media and similar methods to those that have been used for sample analysis in the baseline studies.

## Objectives

The main objective of this EQAS is to support laboratories in assessing and if necessary improve the quality of selective isolation for detection, confirmatory testing and *spa*-typing of MRSA. Furthermore, to assess and improve the comparability of the baseline screening data reported to EFSA by different laboratories within the EU.

## Outline of the MRSA EQAS 2009

### Shipping, receipt and storage of strains

In June 2009 all EU appointed National Reference Laboratories (or the alternative laboratories that have processed the MRSA baseline sample analysis) will receive a parcel from the National Food Institute containing eight dust samples which contain a normal dust background flora and might contain MRSA



(some samples will be negative, or contain other methicillin susceptible or resistant *Staphylococcus* strains).

**NOTE:** The samples should be received and processed immediately upon arrival, or as quickly as possible to assure the stability of the dust samples

## Detection and identification of MRSA from dust samples

### Selective enrichment and isolation

1. In the laboratory, open carefully the tubes containing the dust samples (in a laminar air flow bench and using protective gloves) and add 10 ml of Mueller-Hinton broth supplemented with 6,5 % NaCl to each tube. Mix thoroughly.
2. Incubate at 37 °C for 16-20 h.
3. Take one millilitre of this first pre-enrichment culture and then inoculate into 9 ml Tryptone Soya Broth with 3,5 mg/L cefoxitin and 75 mg/L aztreonam. Mix thoroughly.
4. Incubate for 16-20 h at 37 °C.
5. Take one loop-full (10µl loop) of the latter selective enriched culture and streak onto a Brilliance MRSA Chromogenic Agar plate (Oxoid) and a Blood agar plate.
6. Incubate 24-48 h at 37 °C.
7. Observe the colony morphology (size and coloration) of the colonies obtained on the Brilliance MRSA Chromogenic Agar plate, chose up to five blue colonies indicative for being MRSA and isolate them **from the MRSA selective plate** onto a new blood agar plate. The additional blood agar plate obtained from inoculation of the selective broth is used for parallel observation with the MRSA plate to double-check the colony morphology (to observe typical *S. aureus* morphology and haemolysis), but should not be used for isolation.
8. Incubate the new blood agar plates containing the isolates during 24h at 37°C.



9. Observe the colony morphology of the isolates on the blood agar plate (colour, appearance, haemolysis). Check for purity and re-isolate if necessary.
10. Presumptive isolates of methicillin resistant *Staphylococcus aureus* should at this stage either be stored under appropriate conditions (– 80 °C) for later identification and characterisation or processed immediately.

## Identification of MRSA

Presumptive MRSA isolates should be confirmed as *Staphylococcus aureus* isolates carrying the *mecA* gene by PCR. There is no need to perform other screening methods (such as screening with either oxacillin or ceftioxin) if the isolates have been obtained from selective isolation, thus, the presence of the *mecA* gene can be directly confirmed by PCR amplification. The species identification is simultaneously confirmed by using a multiplex PCR protocol including the amplification of 16S rDNA (internal control of the PCR reaction), nuclease (specific for *Staphylococcus aureus* species) and *mecA*-gene, which should be performed according to the CRL-AR recommended protocol ([http://www.crl-ar.eu/data/images/meca-pcr\\_protocol%2006.02.08.pdf](http://www.crl-ar.eu/data/images/meca-pcr_protocol%2006.02.08.pdf)).

## 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 data submitted, these will be evaluated on the accuracy of the *spa* typing.

## Reporting of results and evaluation

Fill in your results in the enclosed test form. 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 an evaluation report of your results. Please submit results by latest September 1<sup>st</sup> 2009.



If you do not have access to the Internet, or if you experience difficulties entering the data, please return results by e-mail, fax or mail to the National Food Institute.

All results will be summarized in a report which will be made available to all participants. The data in the report will be 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 CRL and the EU Commission. All conclusions are public.

If you have any questions, please do not hesitate to contact the MRSA EQAS Coordinator:

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## How to enter results in the interactive database

Please read this passage before entering the web page. Before you go ahead, you need your test form.

You are able to browse back and forth by using the forward and back keys or click on the CRL logo.

You enter the EU CRL-AR EQAS 2009 start web page (<http://thor.dfvf.dk/crl>) then write your username and password in low cases and press enter. Your username and password is the same as in the previous EQAS's arranged by The National Food Institute. If you have problems with the login please contact us.

Click on "[MRSA tests](#)" to start entering your data regarding the MRSA EQAS.

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, we will ask you to fill in the methods used in a second page which you will reach 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 selective isolation and confirmation of MRSA in this EQAS.

Click on "[Save and go to the next page](#)"

In the data entry pages for each sample "[CRL-MRSA 1.1, ...](#)", you enter the obtained results for each of the MRSA EQAS samples.

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

If you did not perform *spa* typing leave the field blank, in case you have obtained an isolate but it is not a methicillin resistant *Staphylococcus aureus*, choose "not applicable (N/A)", otherwise if you have performed the *spa* typing, choose the *spa* type from the list. Click on "[save and go to next page](#)" to navigate to the next sample results, until you finish to upload all your data.

From the last result sheet you get into the general menu, from where you can review the input pages, approve your input and finally see and print the evaluated results:



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 the evaluated results.

See the evaluated results. You can print each page. *You may have to choose a smaller text size to print the whole screen on one piece of paper.* In the Internet Explorer (or the Internet program you may have), you click on "[view](#)", "[text size](#)" and e.g. "[smallest](#)".



## **Appendix 5 - Test forms MRSA EQAS 2009**

### Test form- MRSA questionnaire

Name:
Name of laboratory:
Name of institute:
City:
Country:
E-mail:
Fax:

***Did you participate in the EU- baseline study for MRSA?***

Yes  / No



How many samples did you process in 2008 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 2008?

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

Which was the origin of the samples processed for MRSA detection in 2008?

- 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:

Any other Comments:



## *TEST FORM – MRSA methods*

Method used for selective isolation of MRSA in this EQAS:

Selective isolation procedure using the exact protocol

different pre-enrichment

Salt concentration used in the Müller Hinton broth: %

Other medium:

different selective enrichment

Cefoxitin concentration: mg/L

Aztreonam concentration: mg/L

Other modifications: medium antimicrobials or concentrations:

different selective agar plates

Different agar brand:

different incubation conditions

Specify: pre enrichment °C/ h;

selective enrichment °C/ h;

selective plating °C/ h



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

- PCR using the CRL recommended multiplex PCR protocol
- modified protocol but same multiplex PCR primers
- other published PCR method
- in- house PCR method

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

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

- PCR using the CRL recommended multiplex PCR protocol
- biochemical methods
- other published PCR method
- in-house PCR method

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



## *TEST FORM*

### **Entry data Sample CRL-MRSA 1.1**

**Please describe the results you have observed regarding this dust sample:**

Growth in pre-enrichment:

Yes / No

Growth in selective enrichment:

Yes / No

Growth in MRSA selective plates:

Yes  / No

**Please describe the growth observed on MRSA selective plates?** (choose only one option)

- Mixed culture containing typical colonies
- Mixed culture without typical colonies
- Pure culture of typical colonies
- Pure culture without typical colonies
- No growth

**Results of confirmatory PCR and species identification:** (choose only one option)

- no isolates tested, sample negative
- mecA* positive, but not *S. aureus* (MRCNS)
- mecA* negative and not *S. aureus* (CNS)



*mecA* negative, *S. aureus* (MSSA)

*mecA* 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

t899

t1333

t1730

t1793

t2510

t2922

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