Report of the 3rd External Quality Assurance System on Isolation, Identification and Typing of Methicillin resistant *Staphylococcus aureus* (MRSA) from Swab Samples, year 2011

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REPORT OF THE 3RD EXTERNAL QUALITY ASSURANCE SYSTEM ON ISOLATION, IDENTIFICATION AND TYPING OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) FROM SWAB SAMPLES, YEAR 2011

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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 (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 for this task by providing assistance in setting up the methods for isolation, identification, and typing of MRSA. Additionally, 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.

In June 2010, the EURL-AR launched an MRSA EQAS trial on dust samples. However, due to stability issues related to the matrix, a second trial was prepared in October 2010, using swab samples. In this year's EQAS, once again swab samples were used within the EQAS trial.

In 2011, we launched the third EQAS performed on MRSA isolation, identification and typing. As the previous EQAS, this included isolation steps besides the identification and also a molecular component; 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 in Europe.

At the starting point of this EQAS, the laboratories should have implemented the methods and enhanced its performance. Therefore, the EQAS may be used to assess the quality of data provided to EFSA, but also to prepare the laboratories for correctly performing the isolation, identification and molecular detection of MRSA.

No thresholds have been set in advance to evaluate the performance of the participating laboratories, nor classify the results of this EQAS as there were issues related to stability which have caused deviations.

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 samples was performed at the Statens Serum Institute (SSI) in Copenhagen, Denmark.

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

A pre-notification (App 1), inviting the participants to the MRSA EQAS 2011 was issued by e-mail to the EURL-AR network on 7th of July 2011 to the NRL-AR's. Additionally, the laboratories that participated in the MRSA baseline studies in 2008 and participated in the MRSA EQAS in 2009 or 2010 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 samples. Participation was free of charge but each laboratory was expected to cover expenses associated with their own analysis.

2.2 Samples

Eight swab 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, methicillin-susceptible *S. aureus* and/or coagulase negative staphylococci strains, besides a mix of *S. aureus*, *Enterococcus faecalis* and *Escherichia coli* which was intended to mimic the normal flora that would be present in a nasal swab collected from an animal.

2.2.1 Preparation of samples:

The background flora was prepared by matching bacterial suspensions of *E. faecium* NS13 (isolate originated from a nasal swab from pig origin); *Staphylococcus sciuri* NS 72 (isolate originated from a nasal swab from pig origin) and *Escherichia coli* ATCC 25922 to a 0,5 Mc Farland standards suspension which were then diluted to 10^{-3} in saline and mixed in equal parts. The swabs were dipped into this mixture before preparation of the samples. The expected amount of each bacterial culture in the mixture was about 10^5 CFU/ml. This was confirmed by performing serial dilution and colony forming unit counts in duplicate from all bacterial suspensions.

The MRSA isolates selected for the MRSA EQAS (EURL-MRSA 3.1- EURL-MRSA 3.8) were prepared in advance and subcultured in week 40-41 and re-subcultured on the day before sample preparation. For the sample standardization, suspensions equal to McFarland 0.5 were prepared in saline tubes of the relevant isolates to contain about 10^8 CFU/mL. The suspensions were further diluted 1:100 or 1:10000 (in the case of the inoculum for samples 3.3 and 3.5) to 10^6 CFU/ml or 10^4 CFU/mL of which 100 µl were used to inoculate the transport media contained in the tubes with the swab samples. The expected inoculum was about 10^5 CFU per sample, for samples 3.1, 3.4, 3.6, 3.7 and 3.8 and was about 10^3 CFU per sample, for samples 3.3 and 3.5 confirmed by performing serial dilution and colony forming unit counts in duplicate from all isolate suspensions. The sample MRSA EQAS 3.2 contained only the background flora and no added test organism.

The strains used for the EQAS were selected from the strain collections at DTU-Food and the identification were 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

SSI, Copenhagen, Denmark. The results obtained after preparation of the contaminated swab 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. Further tests were performed after 5 and 7 weeks using the recommended protocol for isolation and identification of MRSA. The testing revealed that at the time of shipping there was noticeable lack of stability of samples prepared with MRSA EQAS 3.8 and therefore these samples were not sent to the participants for testing. Further stability tests revealed lack of stability in samples MRSA EQAS 3.3 and 3.5 after 2 and 3 weeks after shipment, respectively.

2.3 Isolation and identification of MRSA from swab samples

The protocol for isolation and identification of the MRSA isolates contained in the swab samples was made available on the EURL website (www.EURL-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 in addition to verification of *spa* types at the SSI, 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 also include 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 a week before shipment and were kept at room temperature during the preparation and shipment process. The tubes containing the swab samples in transport media 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 October 24, 2011.

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 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 swab samples in a tube containing transport medium in contrast to large dust swabs. The method recommended for the identification of the isolates was the EURL multiplex PCR which would allow identification of *S. 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 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 25th October, 2011 and closed December 22nd 2011.

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.

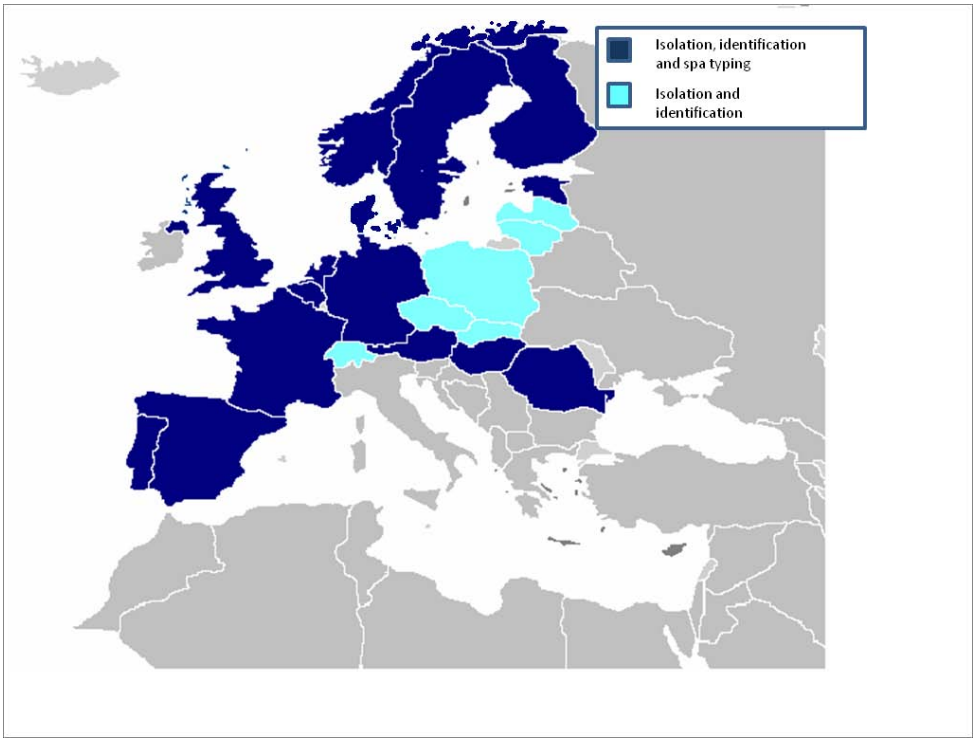
3. Results

A total of 29 laboratories; including 20 NRLs, nine non-NRLs and laboratories from Switzerland and Norway responded to the pre-notification, and were enrolled in the EQAS.

When the deadline for submitting results was reached, 24 laboratories in 21 European countries (including Switzerland and Norway) had uploaded data. One of the NRLs (Lab #21) did not receive the parcel within the time frame for testing, and four laboratories (Labs #30, #37, #40 and #54) did not upload results data due to technical issues. However, one of these (Lab #54) reported the methods used.

One laboratory or more from the following countries provided results to at least one of the EQAS components (also shown below in Figure 1): Austria, Belgium, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Latvia, Lithuania, the Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Spain, Sweden, Switzerland and UK.

Figure 1- Participating Countries



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

3.1 Methods used by EQAS-participants

Of the 24 laboratories submitting results all (100%) of the laboratories participated in the MRSA isolation and identification module whereas only 17 laboratories (71%) 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=19) reported that isolation has been performed according to the protocol provided by the EURL-AR 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 phenol red mannitol broth with 5 mg/L ceftizoxime and 75 mg/l aztreonam. In one lab (Lab #17) the selective medium was modified by using a lower concentration of aztreonam of 50 mg/L and 6% (instead of 6.5%) NaCL was added to the MH-pre-enrichment broth. Different selective plates for the selective isolation, included CHROMagar MRSA (Mast Diagnostica), BD MRSA Chrom Agar (BD); Chrom ID MRSA Agar (bioMérieux) and MRSA Select (Bio RAD).

The species identification of *S. aureus* was performed using the EURL-AR recommended PCR in most laboratories uploading results (n=11) and the other referred methods for identification included other published (n=4) or in-house PCR and RT-PCR methods (n=3) and/or biochemical reactions (n=4) and two did not reply.

The detection of the *mecA* gene was mostly performed by PCR, using the EURL recommended PCR primers (n=15), using other published or In-house PCR methods in the remaining laboratories that reported methods (n=8) and one laboratory did not report the method used.

3.2 MRSA isolation and identification

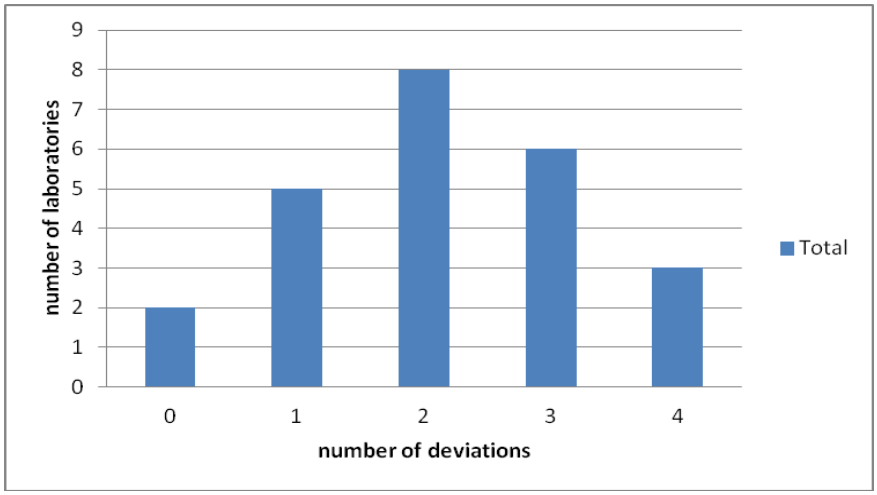
A total of 163 tests (most laboratories have reported results for all the seven samples and only Lab #28 uploaded results for only two samples) have been performed and the overall result indicate that 112 tests were correct, corresponding to 82%. All 69 samples expected to be negative were correctly assigned, however we have observed a rather large number of deviations in the samples expected to be positive.

Table 1-. The overall performance of MRSA isolation and identification, 2011.

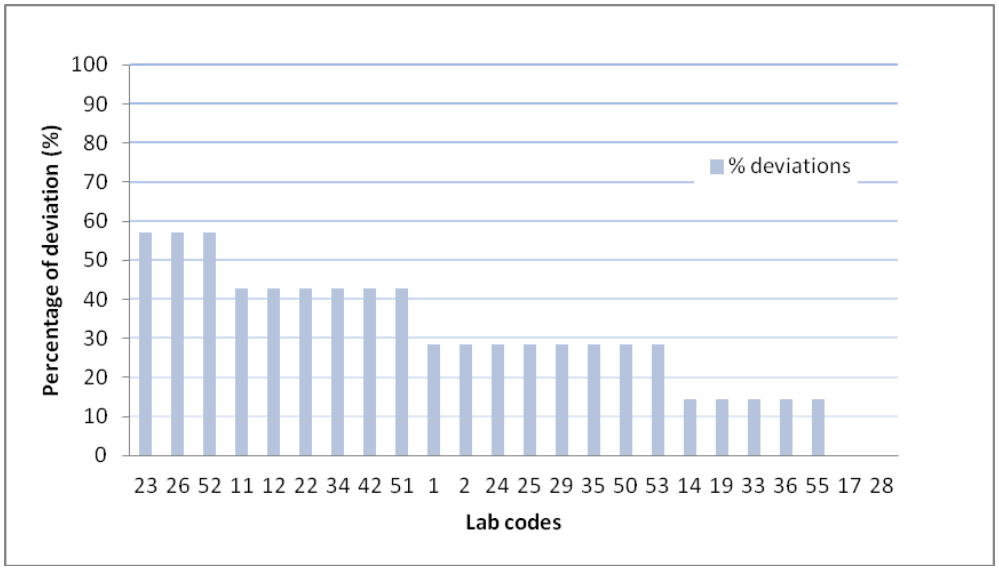
Isolation of MRSA from swab samples		**Correctly classified samples**	
Number of performed tests		Number of correct tests N(%)	
n	%	N	%
163	100	112	82
Number of expected negative tests		Number of correctly identified negative tests	
n	%	N	%
69	42	69	100
Number of expected positive tests		Number of correctly identified positive tests	
n	%	N	%
94	58	43	46

In general, the individual laboratory results of this MRSA EQAS on isolation and identification of MRSA from swab samples, showed a good specificity by most laboratories. In fact, all laboratories providing results classified correctly the expected negative swabs provided and no false positives were observed. However, the sensitivity was quite low and a large number of the samples expected to be positive were misclassified. Only one laboratory was able to correctly assign all the seven tested samples (Lab #17). The remaining laboratories had between one and four deviations, among the seven samples shipped (Graph 1). One laboratory, Lab #28 has only submitted the final interpretation for two of the positive samples (EURL-MRSA 3.6 and EURL-MRSA- 3.7) and has therefore only reported two correct results, although no deviating results were reported.

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



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



As described above, all deviations were caused by a lack of sensitivity of the methods used, leading to false negative results in 51 samples in total. The lower level of inoculation for all samples is probably the main cause of the deviations in general as the level was reduced to 10^5 CFU/mL in samples EURL-MRSA-3.6 and EURL-MRSA-3.7 and 10^3 CFU/mL for samples EURL-MRSA-3.3 and EURL-MRSA-3.5. The latter samples inoculated with very low inoculums were just around the detection limit and

might have become unstable 1-2 weeks after shipping (as observed under our stability assays), which might have caused most of the deviations observed for these samples.

As referred before, Only Lab# 17 showed no deviations in the seven samples, and Lab #28 Had no deviations in the results reported but has only submitted the final interpretation for two of the positive samples (EURL-MRSA 3.6 and EURL-MRSA- 3.7). Among the 22 laboratories reporting deviating results five laboratories reported one deviation, eight laboratories reported two deviations, six laboratories reported three deviations and three laboratories (Lab #23, #26 and #52) have reported four deviating results. The latter laboratories (Lab #23, #26 and #52) had assigned a negative result for MRSA to all samples tested and were not able to recover any of the MRSA in the samples.

An internal control was prepared in order to have one strain in common to both MRSA EQAS 2010 and 2011 trials. The internal control was expected to be shipped within sample EURL-MRSA 3.8, which contained the same MRSA *spa* t075 strain sent the previous year as EURL-MRSA 2B.7. However, due to last minute problems with stability, this sample was not shipped to the NRL's and therefore we will not be able to compare results over time, for the internal control, in this trial.

3.3 *spa* typing of the MRSA strains

The *spa* typing module accounted participation of 17 laboratories, which have uploaded *spa* typing data.

The reported results for *spa* typing included 80 tests and the general results show that 57 (71%) of results were considered correct whereas 23 (29%) deviation were observed. One laboratory (Lab #17) was able to correctly assign all the seven samples, other five laboratories did not have any deviations either, however, these laboratories uploaded results for two or three of the positive samples only.

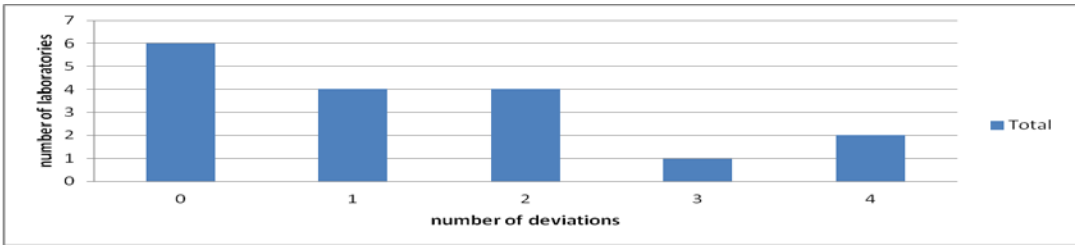
In this module, samples EURL-MRSA-3.3, EURL-MRSA-3.5, EURL-MRSA-3.6 and EURL-MRSA-3.7 were identified positive for MRSA. Therefore, optional *spa* typing was offered for the respective isolates found, whereas the remaining negative samples EURL-MRSA-3.1, EURL-MRSA-3.2 and EURL-MRSA-3.4 would not render a positive MRSA isolate for typing and therefore be classified as N/A (not applicable).

Some of the laboratories have only reported the results regarding the sample previously found positive for MRSA, whereas no more than eight have reported N/A results for all the samples considered MRSA negative, as required. In this way the range of uploaded *spa* types diverged from one to seven samples, uploaded by each participating lab.

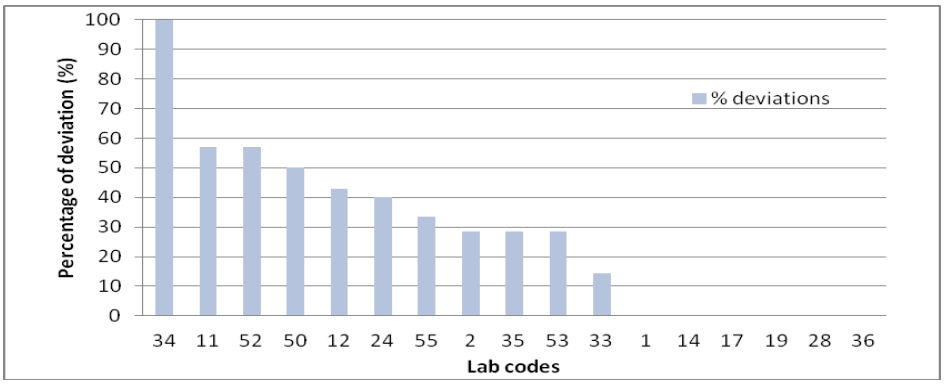
By laboratory, the results show that six laboratories did not have any deviations. However, most laboratories uploading *spa* typing data have obtained some deviations (ranging from 0-4 deviations per lab), mostly related to the lack of isolation of the expected MRSA (false negatives, which were therefore not *spa* typed). Lab #34 has only uploaded results for *spa* typing of EURL-MRSA 3.7 which was incorrectly assigned, and has therefore obtained 100% deviation, however representing one sample only.

Most of the deviations were caused by lack of isolation of the expected MRSA isolates and uploading a *spa* typing result as N/A, and therefore not related to incorrect *spa* type assignment (n=19). Three deviations due to wrong *spa* type assignment were observed in three laboratories (Lab #34, #50 and #55).

Graph 3- Number of deviations per laboratory for *spa* typing.



Graph 4- Deviating results for *spa* typing, per participating laboratory. The laboratories were ranked by decreasing percentage of deviation.



Note: Lab #34 has only uploaded results for one *spa* type which was found wrong, therefore, showing a 100% deviation, even though it represents only one sample. In a similar way Lab #50 and #55 which obtained a deviation percent of 50% and 33% had actually uploaded results for two and three samples respectively, one of which was found deviating.

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

Sample number	N participating laboratories	Repeat succession	expected *spa* type	correct	Deviating results (number of deviations)
EURL-MRSA 3.1	9	None	N/A	9	None
EURL-MRSA 3.2	9	None	N/A	9	None
EURL-MRSA 3.3	12	07-16-23-02-12-23-02-34	t1430	5	N/A (7)
EURL-MRSA 3.4	9	None	N/A	9	None
EURL-MRSA 3.5	10	08-16-02-25-02-25-34-24-25	t034	2	N/A (8)
EURL-MRSA 3.6	15	08-16-02-25-34-24-25	t011	12	N/A (2) t108 (1)
EURL-MRSA 3.7	16	08-16-02-25-24-25	t108	11	N/A (3) t021 (1) t1430 (1)
EURL-MRSA 3.8	None *	11-19-21-21-12-21-17-34-24-34-22-25	t075	None *	-

* not shipped due to stability issues.

N/A- not applicable

4. Discussion

4.1 MRSA isolation and identification

In the 2011 EURL-AR MRSA EQAS trial, the samples were prepared with strains selected based on recent findings and the quantity inoculated was reduced 10 times for the samples EURL-MRSA 3.6 and 3.7 and reduced 1000 times for samples EURL-MRSA 3.3 and 3.5. This reduction in inoculums has changed the results dramatically from last year's EQAS, increasing the number of deviations due to lack of sensitivity in retrieving the MRSA present in the sample. Interestingly, the only laboratory being able to identify all suspected positive samples correctly (lab #17) has used a slightly modified isolation method (6 % NaCl added to the pre-enrichment broth and 50 mg/l aztreonam added to selective enrichment broth).

This lack of sensitivity and the problems observed with lack of stability of the samples after shipment lead to a high number of deviations in the isolation/identification trial, especially regarding the samples with low level inoculums. However, as previously the MRSA negative results were correctly assigned indicating that the isolation/identification methods were highly specific.

4.2 *spa* typing

For *spa* typing, 17 laboratories participated in the trial which indicates and increase in participation. The results uploaded were good, with six of the 17 laboratories showing no deviation. The range of uploaded results by the participating laboratories was very broad and most deviating results were caused by lack of isolation of the MRSA, as only three deviations were caused by wrong *spa* type assignment. Most deviations observed do not demonstrate any problems with the *spa* typing method, but reflect only a small issue concerning possible cross contamination or sample management that might have caused the deviation observed in two of the *spa* types found incorrect (found *spa* type could be from MRSA in another sample from this EQAS) where as another found *spa* type (t021) was not supposed to be found among the MRSA that could be isolated from the samples in this EQAS, indicating other source of contamination or sample switch as the expected *spa* sequences are relatively different from those of the *spa* type found.

5. Conclusion

In general, the results of the third MRSA EQAS 2011 demonstrate that most participating labs have set up the methods and are able to isolate MRSA from swab samples in a reliable fashion. Furthermore, the identification of MRSA using molecular methods is performed successfully in most the participating laboratories, with excellent specificity, but still needs for improvement of the test sensitivities, especially when the MRSA contamination is at very low level.

The participation in the *spa* typing module has increased indicating that more laboratories have this method available among the laboratories in Europe. However, still seven laboratories participating in the MRSA EQAS 2011 did not take part in this optional module. This might indicate that these laboratories have difficulties in performing *spa* typing and/or have not set up this methodology.

The results of this 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. Most deviations observed in this module were due to lack of isolation of the MRSA and therefore as a result of false negative results. The three deviations observed which were due to wrong *spa* type assignment might be due to possible switching of samples or cross contamination between samples within the EQAS samples, and in one case maybe of other samples, 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

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- EURL-AR MRSA EQAS 2011 pre-notification

EXTERNAL QUALITY ASSURANCE SYSTEM (EQAS) 2011 FOR DETECTION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN SWAB SAMPLES

The EURL are pleased to announce the launch of the third MRSA-EQAS trial. 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 samples which contain a background flora and of which some will contain MRSA. The samples will be swabs in tubes containing transport medium

This EQAS is specifically for designated NRL-ARs and additional designated laboratories performing the selective isolation and identification of MRSA from pig farms. These laboratories therefore 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 regarding the contact information. If no laboratory from your country has participated in the MRSA EQAS 2010, you are welcome to contact us to sign up for the participation in this EQAS. Participation is free of charge for all above-mentioned designated laboratories.

TO AVOID DELAY IN SHIPPING THE ISOLATES TO YOUR LABORATORY

Please remember to provide the EQAS coordinator with documents or other information that can simplify customs procedures (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 content of the parcel is “Biological Substance Category B”.

TIMELINE FOR RESULTS TO BE RETURNED TO THE NATIONAL FOOD INSTITUTE

Shipment of isolates and protocol: The samples will be shipped in October 2011. The protocol will be provided via our website. This protocol, as in the previous trials will be based on the protocol used in the baseline studies and therefore we would like to ask you to have the same 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 December 16th 2011. 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: 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) or by fax (+45 32 88 63 41).

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			National Diagnostic and Research Veterinary Institute	Bulgaria
X	X		SVI Olomouc	Czech Republic
X	X	X	The National Food Institute	Denmark
X	X	X	Estonian Veterinary and Food Laboratory	Estonia
X	X	X	Finnish Food Safety Authority EVIRA	Finland
X	X	X	ANSES (French Agency for Food, Environmental and occupational Health & Safety).	France
X	X	X	Federal Institute for Risk Assessment	Germany
X	X	X	Central Agricultural Office, Veterinary Diagnostical Directorate	Hungary
X			Istituto Zooprofillatico Sperimentale Delli Regioni Lazio e Toscana	Italy
X	X		National Diagnostic Centre of Food and Veterinary Service	Latvia
X	X		National Food and Veterinary Risk Assessment Institute	Lithuania
X	X	X	Animal Health Service Deventer	Netherlands
X	X		Central Veterinary Institute of Wageningen	Netherlands
X	X	X	Faculty of Veterinary Medicine Utrecht	Netherlands
X	X	X	Food and Consumer Product Authority (VWA)	Netherlands
X	X	X	Veterinærinstituttet	Norway
X	X		National Veterinary Research Institute	Poland
X	X	X	Faculdade de Medicina Veterinária- UTL	Portugal
X			Laboratório Nacional de Investigacão Veterinária (LNIV)	Portugal
X	X	X	National Institute of Research	Romania
X			Institute of Veterinary Medicine of Serbia	Serbia
X	X		State Veterinary and Food Institute (SVFI)	Slovakia
X			National Veterinary Institute	Slovenia
X	X	X	Complutense University of Madrid	Spain
X	X	X	National Veterinary Institute, SVA	Sweden
X	X		Vetsuisse faculty Bern, Institute of Veterinary Bacteriology	Switzerland
X	X	X	The Veterinary Laboratory Agency	United Kingdom

Appendix 3- expected results MRSA EQAS 2011

Sample ID	*mecA*	*spa* type	MRSA status sample
MRSA EQAS- 3.1	positive	N/A	**Negative (MRCNS)**
MRSA EQAS- 3.2	N/A	N/A	**Negative (Blank)**
MRSA EQAS- 3.3	positive	t1430	**Positive**
MRSA EQAS- 3.4	negative	N/A (t034)	**Negative (MSSA)**
MRSA EQAS- 3.5	positive	t034	**Positive**
MRSA EQAS- 3.6	positive	t011	**Positive**
MRSA EQAS- 3.7	positive	t108	**Positive**
MRSA EQAS- 3.8*	positive	t075	**Positive (internal control)**

*Sample MRSA EQAS-3.8 was not shipped due to stabilty problems in the samples.

N/A- not applicable

Appendix 4- Protocol for the MRSA EQAS 2011

Protocol for MRSA EQAS 2011

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

Introduction

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

As referred in the pre-notification, this EQAS is designed specifically for designated NRL-ARs and additional designated laboratories performing the selective isolation and identification of MRSA from pig farms.

In this MRSA EQAS we have prepared swab samples, which should be processed in a similar way as in the previous EQAS 2010, according to the following description in this protocol.

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 2011

Shipping, receipt and storage of strains

In October 2011 all EU appointed National Reference Laboratories (or the alternative laboratories that have processed the MRSA baseline sample analysis) will receive a parcel from the EURL-AR containing eight swab samples which contain a 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 samples.

Detection and identification of MRSA from swab samples

Selective enrichment and isolation

1. In the laboratory, open carefully the tubes containing the swab samples in transport medium (in a laminar air flow bench and using protective gloves) and inoculate each swab into a tube containing 10 ml of Mueller-Hinton broth supplemented with 6.5 % NaCl. 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, choose 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 37ºC.
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 cefoxitin) 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 EURL-AR recommended protocol (http://www.eurl-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 a submission report of your results. Please submit results by latest December 16th 2011. After the deadline, the database will be closed and you will be able to view and print an automatically generated report evaluating your results.

If you experience difficulties entering the data, please return results by e-mail, fax or mail to the EURL-AR.

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 EURL-AR 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 EURL logo.

You enter the EURL-AR EQAS 2011 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 carefullly 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 “EURL-MRSA 3.1, …”, 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 submitted results.

Appendix 5 – Example of test forms MRSA EQAS 2011

Test forms MRSA EQAS 2011

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 2010 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 2010?

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

Which was the origin of the samples processed for MRSA detection in 2010?

- ☐ 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 enrichment, agar plates or incubation conditions

If you used different pre enrichment please indicate:

Salt concentration used in the Müller Hinton broth: %

Other medium:

If you used different enrichment please indicate

Cefoxitin concentration: mg/L

Aztreonam concentration: mg/L

Other modifications: medium antimicrobials or concentrations:

If you used different selective agar plates

Different agar brand**:**

If you used 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 EURL 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 EURL recommended method and justify your choice**:**

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

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

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

TEST FORM
Entry data Sample EURL-MRSA 3.1

Please describe the results you have observed regarding this swab 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
☐ t1430
☐ t1730
☐ t1793
☐ t2510
☐ t2922
☐ t899

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