CODA
CENTRUM VOOR ONDERZOEK IN DIERGENEESKUNDE EN AGROCHEMIE

# MRSA surveillance 2013: Pigs

1. Introduction

In the framework of the FASFC surveillance, a surveillance of MRSA in pigs has been executed in order to determine the prevalence and diversity of MRSA in pigs for the year 2013. For this surveillance, the European Food Safety Authority (EFSA) proposed standardized protocol for the isolation of MRSA from dust samples was used in order to obtain comparable prevalence results all over Europe. However, this protocol was estimated not to be most sensitive during the previous surveys in poultry in 2011 and in bovines 2012.

2. Materials and methods

2.1. Sample origin

Three-hundred twenty-eight farms were sampled during 2013. 20 nasal swabs per farm were taken.

2.2. Isolation methods

For all of the 328 farms, the 20 nasal swabs were pooled per farm and incubated in Mueller-Hinton (MH) broth (Becton Dickinson) supplemented with NaCl (6.5%) at 37°C for 20-24h. One ml of this broth was added to Tryptic Soy Broth (TSB) supplemented with cefoxitin (3.5mg/l) and aztreonam (75mg/l) and incubated at 37°C overnight. Ten microliter of this broth was then plated on MRSA-ID (biomérieux) and incubated 48 hours at 37°C. At both 24 and 48 hours, plates were inspected and suspected colonies were purified on a Columbia Sheep Blood (CSB) agar plate and incubated overnight at 37°C.

2.3. DNA extraction

One colony from the CSB plate was inoculated in 1ml of BHI and incubated for 18-24 hours at 37°C. The cultures in Eppendorf tubes were then centrifuged for 2.5 minutes at 13500 rpm in an Eppendorf centrifuge. Supernatant was discarded and cells were washed with sterile water and another centrifugation step was done. Subsequently, the supernatant was discarded. Five µl of lysostaphin (1mg/ml) was then mixed with 45 µl of distilled water and added to the pellet. This suspension was incubation at 37°C for 10 minutes. Following the addition of 45µl of distilled water, 5µl of proteinase K (2mg/ml) and 150µl of tris-HCl (0.1M, pH8), the suspension was incubated at 60°C for ten minutes and then heated at 100°C for five minutes. This suspension was centrifuged for five minutes in an Eppendorf centrifuge at 13500 RPM. Supernatants was collected and stored at -20°C for further analysis.

2.4. MRSA identification

MRSA identification was performed using a triplex PCR, previously published by Maes *et al*. 2002. This PCR allows detecting the staphylococcal specific 16S rDNA gene , the *nuc1* gene

specific for *S.aureus*, and the presence of the *mecA* gene responsible for methicillin resistance. The Triplex PCR reaction mixture consisted of 6.25µl of MasterMix (Qiagen), 0.75µl of 16S rRNA primers and 0.5µl of *mecA* and *nuc* primers (concentration 10µM), 0.75µl of RNAse free water and 2µl of sample. The PCR consisted of a first denaturation step (94°C , 10 minutes) followed 35 cycles of denaturation (94°C, 60 seconds), annealing (51°, 60 seconds) and elongation (72°C, 120 seconds) and by a final elongation step (72°C, 5 minutes). The obtained products were separated in a 2% agarose gel and run at 70V for one hour. The 100bp DNA-ladder was used as a size standard (O'range ruler, Fermentas).

2.5. Genotyping

2.5.1.*Spa* typing

All MRSA isolates were *spa*-typed by sequencing the repetitive region of the *spa* gene encoding for the staphylococcal protein A. This method depicts the rapid evolution, since through recombination, the repeats may change fast. The protein A (*spa*) gene was amplified according to the Ridom StaphType standard protocol (**www.ridom.de/staphtype**) and the amplification was checked on a 2% agarose gel. Sequencing was performed with CEQ 8000 using standard protocols and sequences were compared with the international Ridom database.

2.5.2.CC398 PCR

CC398 PCR was performed on all MRSA following protocol described by Stegger *et al.* 2011. This method allows the rapid detection of the *S. aureus* sequence type ST398.

2.5.3.Multi Locus Sequence Typing (MLST)

MLST typing was performed on MRSA isolates that were negative in the CC398 PCR and/or showed a spa type that has never been associated with CC398 before. The protocol used was the internationally accepted protocol described by Enright *et a.,* 2000. The allelic profile of the *S. aureus* strains were obtained by sequencing internal fragments of seven house-keeping genes: *arcC* (Carbamate kinase), *aroE* (Shikimate dehydrogenase), *glpF* (Glycerol kinase), *gmk* (Guanylate kinase), *pta* (Phosphate acetyltransferase), *tpi* (Triosephosphate isomerase), *yqil* (Acetyle coenzyme A acetyltransferase). Sequences of internal fragments were then compared to the international database (**http://saureus.mlst.net**) to obtain the sequence type.

2.6. Determination of antimicrobial resistance in MRSA strains by micro-dilution (Sensititre ®)

Antimicrobial resistance was determined using the micro broth dilution method (Sensititre, Trek Diagnisis Systems, Magellan Biosciences) following the manufacturer's instructions and using the EUCAST ECOFF breakpoints for *S. aureus*. The antibiotics tested were those included in the EUST custom panel plate for staphylococcus: chloramphenicol (CHL; [4mg/l – 64mg/l]), ciprofloxacin (CIP; [0.25mg/l – 8mg/l]), clindamycin (CLI; [0.12mg/l – 4mg/l]), erythromycin

(ERY; [0.25mg/l – 8mg/l]), cefotoxin (FOX; [0.5mg/l – 16mg/l]), fusidic acid (FUS; [0.5mg/l – 4mg/l]), gentamicin (GEN; [1mg/l – 16mg/l]), kanamycin (KAN; [4mg/l – 64mg/l]), linezolid (LZD; [1mg/l – 8mg.l]), mupirocin (MUP; [0.5mg/l – 256mg/l]), penicillin (PEN; [0.12mg/l – 2mg/l]), rifampicin (RIF; [0.016mg/l – 0.5mg/l]), sulfamethoxazole (SMX; [64mg/l – 512mg/l]), streptomycin (STR; [4mg/l – 32mg/l]), quinupristin/dalfopristin(SYN; [0.5mg/l – 4mg/l]), tetracycline (TET; [0.5mg/l – 16mg/l]), tiamulin (TIA; [0.5mg/l – 4mg/l]), trimethoprim (TMP; [2mg/l – 32mg/l]), vancomycin, (VAN ; [1mg/l – 16mg/l]). Samples were first inoculated on a blood agar plate and incubated at 37°C for 24 hours. Three to five colonies from the agar plate were then added in 4 ml of sterile water and adjusted to 0.5 McFarland. Ten microliter of this suspension was inoculated in a tube containing 11ml cation adjusted MuellerHinton broth with TES (Trek Diagnostics). Fifty µl of this inoculum was then inoculated per well using the AIM™ Automated Inoculation Delivery System and incubated at 37°C for 24 hours**.** Sensititre plates were read with Sensititre Vision System® for semi-automatic registration of the Minimum Inhibitory Concentration (MIC) of the different antimicrobials tested. The MIC was defined as the lowest concentration by which no visible growth could be detected.

### 2.7. Statistical analysis

The number of resistant strains was counted and resistance percentages were calculated. Exact confidence intervals for the binomial distribution were calculated using a visual basic application in Excel. A 95% symmetrical two-sided confidence interval was used with p=0.025. The lower and upper bound of confidence interval for the population proportion was calculated. Based on the Pearsons chi-square test, and where appropriate the Fischer exact test, significance of the differences were calculated.

## 3. Results

### 3.1. Prevalence

Two-hundred fifteen samples (65.6%, 95% CI [60.1% - 71%]) were positive for MRSA.

### 3.4. Antimicrobial resistance

Antimicrobial susceptibility was tested on 211 strains, four strains were lost.
As expected, all strains were resistant to penicillin. One strain is susceptible to cefoxitin, but this should be regarded as a methodological deviation since the presence of the *mecA* gene was demonstrated. It is know that MIC testing for MRSA is not a 100% sensitive. More than 90% of the strains were resistant to tetracycline (98.1%) and trimethoprim (95.7%). A high prevalence of resistance was also observed to ciprofloxacin (61.1.0%), clindamycin (66.4%),

erythromycin (57.3%). kanamycin (43.1%) and gentamicin (45.5%) resistance was present in a little less than half of the strains. Lower resistance levels were detected to streptomycin (30.8%) fusidic acid (20.4%), sulfamethoxazole (27.5%), quinupristin/dalfopristin (27.5%), tiamulin (32.7%), rifampicin (19.0%). Resistance to chloramphenicol (10.9%) and mupirocin (10.0%) were around 10%. No resistance were observed to vancomycin (Table 4). One strain was classified as resistant to linezolid, however, the MIC was close to the breakpoint and this should be confirmed by other methods.

Multi-resistance was calculated and results are shown in table 6. It should be noted that all but one strain are resistant to at least 2 antibiotics, cefoxitin and penicillin since they are a selection of MRSA. As such 50% of the strains are at least resistant to 7 different antibiotics. Seven strains had resistance to as much as 17 different antibiotics, remaining susceptible only to linezolid and vancomycin. These strains were all MRSA CC398 t011. The strains resistant to 15 or 16 antibiotics were all but two t011, one strain was a t034 and another strain was t2370. All were CC398. The strains were additionally susceptible to chloramphenicol and/or ciprofloxacin and/or erythromycin and/or mupirocin. Of the 5 strains resistant to 14 antibiotics one was a t1456 strain and the other strains were t011.
The one strain resistant to 3 antibiotics was additionally resistant to tetracycline and was a t011 strain. The 5 strains resistant only to 4 antibiotics were a t4150 strain, a strain that has been detected before in poultry and bovines and was found to be a ST239, a hospital associated MRSA. This strain was phenotypically classified as susceptible. Three other strains were CC398 t011 and one strain was t044 strain of which the sequence type was not determined.

| Dilution | Antibiotic and number of strains with MIC | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FUS | CHL | CIP | ERY | FOX | GEN | LZD | Str | SYN | TET | VAN | KAN | MUP | PEN | RIF | SMX | CLI | TIA | TMP |
| <=0.016 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 171 | 0 | 0 | 0 | 0 |
| 0.03 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| 0.06 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| 0.12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 63 | 0 | 0 |
| 0.25 | 0 | 0 | 38 | 34 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 8 | 0 | 0 |
| 0.5 | 168 | 0 | 26 | 51 | 0 | 0 | 0 | 0 | 109 | 3 | 0 | 0 | 170 | 1 | 2 | 0 | 5 | 136 | 0 |
| 1 | 2 | 0 | 18 | 5 | 0 | 95 | 23 | 0 | 44 | 1 | 207 | 0 | 20 | 0 | 33 | 0 | 2 | 5 | 0 |
| 2 | 8 | 0 | 23 | 8 | 0 | 20 | 184 | 0 | 21 | 0 | 4 | 0 | 6 | 3 | 0 | 0 | 6 | 1 | 9 |
| 4 | 28 | 4 | 9 | 0 | 1 | 14 | 3 | 61 | 22 | 0 | 0 | 103 | 0 | 205 | 0 | 0 | 5 | 5 | 2 |
| 8 | 5 | 146 | 56 | 1 | 57 | 24 | 1 | 62 | 15 | 0 | 0 | 17 | 0 | 0 | 0 | 0 | 122 | 64 | 1 |
| 16 | 0 | 38 | 41 | 112 | 89 | 27 | 0 | 23 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 32 | 0 | 7 | 0 | 0 | 64 | 31 | 0 | 13 | 0 | 207 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 64 | 0 | 15 | 0 | 0 | 0 | 0 | 0 | 52 | 0 | 0 | 0 | 14 | 0 | 0 | 0 | 138 | 0 | 0 | 198 |
| 128 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 69 | 0 | 0 | 0 | 15 | 0 | 0 | 0 |
| 256 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 15 | 0 | 0 | 7 | 0 | 0 | 0 |
| 512 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 |
| 1024 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 49 | 0 | 0 | 0 |
| %R | 20,4 | 10,9 | 61,1 | 57,3 | 99,5 | 45,5 | 0,5 | 30,8 | 27,5 | 98,1 | 0,0 | 43,1 | 10,0 | 100,0 | 19,0 | 27,5 | 66,4 | 32,7 | 95.7 |
| CI | 15,2-26 | 17,0-16,0 | 54,2-68 | 50,4-64 | 97,4-100 | 38,6-52 | 0-3 | 24,6-38 | 21,6-34 | 95,2-99 | 0-2 | 36,3-50 | 6,3-15 | 98,3-100 | 13,9-25 | 21,6-34 | 59,5-73 | 26,4-39 | 92.1-98 |

Table 1. MICs of 211 methicillin-resistant *S.aureus* tested with micro-broth dilution. Values in grey cells mean that the MIC was higher than the highest concentration tested. Epidemiological cut-off value is indicated with the line in bold.

| N antibiotics | N strains | % of strains | Cumulative % of strains |
|---|---|---|---|
| 0 | 0 | 0,0 | 0,0 |
| 1 | 0 | 0,0 | 0,0 |
| 2 | 0 | 0,0 | 0,0 |
| 3 | 1 | 0,5 | 0,5 |
| 4 | 5 | 2,4 | 2,8 |
| 5 | 37 | 17,5 | 20,4 |
| 6 | 31 | 14,7 | 35,1 |
| 7 | 36 | 17,1 | 52,1 |
| 8 | 26 | 12,3 | 64,5 |
| 9 | 22 | 10,4 | 74,9 |
| 10 | 7 | 3,3 | 78,2 |
| 11 | 7 | 3,3 | 81,5 |
| 12 | 4 | 1,9 | 83,4 |
| 13 | 2 | 0,9 | 84,4 |
| 14 | 5 | 2,4 | 86,7 |
| 15 | 14 | 6,6 | 93,4 |
| 16 | 7 | 3,3 | 96,7 |
| 17 | 7 | 3,3 | 100,0 |
| 18 | 0 | 0,0 | 100,0 |
| 19 | 0 | 0,0 | 100,0 |

Table 2. Multi-resistance in MRSA from pigs.

### 3.5. MRSA characterization

Among 215 MRSA isolates recovered, 11 strains were excluded due to insufficient typing, not allowing to make concise conclusion on the strains. Two of these were excluded based on an invalid spa type. Six were excluded because of the absence of a sequence type associated. The rest was excluded because the CC398 PCR did not match the spa type. The remaining 204 strains were analysed and will be described underneath. Results are summarised in table 3.

Table 3. Strain types

| Spa type | MLST | Number of strains |
|---|---|---|
| t011 | 398 | 180 |
| t034 | 398 | 6 |
| t044 | 80 | 2 |

| | | |
|---|---|---|
| t1451 | 398 | 1 |
| t1456 | 398 | 2 |
| t1580 | 398 | 2 |
| t1985 | 398 | 2 |
| t2123 | 398 | 1 |
| t2370 | 398 | 2 |
| t3171 | 398 | 1 |
| t4150 | 239 | 1 |
| t4432 | 398 | 1 |
| t4872 | 398 | 1 |
| t6228 | 398 | 1 |
| t8100 | 398 | 1 |

Four strains that were negative in the CC398 PCR were a t034 strain (confirmed ST398 by MLST), a t4150 strain that has been shown to be associated with ST239 and two t044 strains that are associated with ST80 as determined by MLST. It can thus be concluded that of the 204 fully typed strains, three were not CC398. The Sequence type ST 239 has been found before in the surveillance in poultry in 2011, however, then it was a spa type t037-ST239. ST239 is a HA-MRSA and the spa type t4150 has rarely been reported, and this mainly in Asia and New Zealand. ST80 belongs to the community acquired MRSA and is considered the European CA-MRSA clone, but has been found also in the Middle East and North Africa. ST80, like also ST239 has been associated with multiple spa types.

Amongst the CC398 strains, 13 different spa types were found. The vast majority were however the commonly isolated t011 and t034.

## 4. Conclusions

Compared to former studies on the prevalence of MRSA in pigs in Belgium (2007 and 2009), there are no changes in prevalence, when you take into account that the 2009 Europeans study by EFSA was less sensitive as the current study.

While before, few spa-types were found now, there is a larger diversity than ever detected before. However, it should be noted also that in this study, the number of strains isolated was far higher than before due to the larger sample, allowing to have a better view on the diversity.

As in poultry and bovines, also in pigs, human associated serotypes were detected. In pigs, both a HA-MRSA and a CA-MRSA were detected. This indicates that we vigilance for the acquisition of virulence genes by MRSA CC398. If this clone is becoming more virulent, it may have major implications for both human and animal health.

Finally, and as has been demonstrated before, the CC398 clones are highly multi-resistant. The only antibiotic for which no resistance has been found was the glycopeptide antibiotic vancomycin.

5. References

**Enright M.C., DAY N.P.J., Davies C.E., Peacock S.J., Spratt B.G.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus.* Journal of Clinical Microbiology. **38**: 1008-1015.