CODA
CENTRUM VOOR ONDERZOEK IN DIERGENEESKUNDE EN AGROCHEMIE

MRSA surveillance 2012: Bovines

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1. Introduction

In the framework of the FASFC surveillance and the EMIDA-ERA NET project, a surveillance of MRSA in bovines has been executed in order to determine the prevalence and diversity of MRSA in bovines for the year 2012. 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 very sensitive during the previous survey in poultry in 2011. Since bovines and especially veal calves were expected to have a higher prevalence, we compared the single broth enrichment method (SBEM) with the double broth enrichment method (DBEM) to determine whether there were differences between the two methods in this higher prevalence population.

2. Materials and methods

2.1. Sample origin

Four hundred and thirty-two farms were sampled during 2012. Of these, 141 were dairy farms, 187 farms reared beef cattle and 104 reared veal calf. Twenty animals per farm were sampled using nasal swabs.

2.2. Isolation methods

2.2.1. Double broth enrichment method (DBEM)

For all of the 432 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.

2.2.2.Single broth enrichment method (SBEM)

The alternative protocol was applied on samples of 106 farms and was similar to the DBEM protocol, with the sole difference the omission of the second enrichment in antibiotic supplemented broth.

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 only performed on MRSA isolates that were negative in the CC398 PCR. 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 – 8mg/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.

The Cohen's kappa coefficient (2x2 table) was calculated in order to compare both isolation methods. Cohen's kappa coefficient was interpreted as shown in the table 1 (Landis and Koch, 1977[4]). This analysis includes the first 106 farms. DBEM is considered as the Gold standard while SBEM is the one under estimation.

K-value	Interpretation
≤ 0	No agreement
0.01-0.20	Slight agreement
0.21-0.40	Fair agreement
0.41-0.60	Moderate agreement
0.61-0.80	Substantial agreement
0.81-1.00	Almost perfect agreement

Table 1: Interpretation of Cohen's kappa coefficient

Cohen's Kappa coefficient, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of both methods were also calculated using the previously described formulae (Dohoo *et al*., 2009) and Win Episcope 2.2.

3. Results

3.1. Prevalence

By using the official DBEM, 82 samples (19.0%, 95% CI [15.3% - 22.7%]) were positive for MRSA. Among these farms, 14 (9.9%, 95% CI [5.0% - 14.9%]) were dairy farms, 19 (10.2%, 95% CI [5.8% - 14.5%]) were farms holding meat cows and 49 were (47.1%, 95% CI [37.5% - 56.7%]) farms raising veal calves were positive for MRSA. Since one sample has been lost after identification, antimicrobial resistance and molecular characterization is given for 81 samples.

Farm type	N samples	N positive	% positive	Confidence interval
Dairy	141	14	9.9	5.3-22.7
Beef cattle	187	19	10.2	5.0-14.9
Veal calves	104	49	47.1	37.5-56.7

Table 2. Prevalence of MRSA in the different animal categories.

3.2. Comparison of isolation methods

Comparisons were performed on 106 samples. Using both isolation methods (Table 3), 34 (32.1%, 95% CI [23.2-41.1]) farms out of 106 tested were found to be positive. Because, nine farms were detected positive with the SBEM but not with the DBEM and conversely, nine other farms were detected positive with the DBEM but not with the SBEM, Kappa agreement coefficient (k) was 0.61. This indicates a substantial agreement between both methods. There was no significant difference between the prevalence of these methods (P> 0.05). Specificity, positive predictive value and negative predictive value were likewise identical (Table 4).

		DBEM		Total
		Positive	Negative	
SBEM	Positive	25	9	34
	Negative	9	63	72
Total		34	72	106

Table 3. Comparison of methicillin-resistant *Staphylococcus aureus* isolates detected using Double Broth Enrichment Method (DBEM) or Single Broth Enrichment Method (SBEM).

	DBEM (%)	SBEM (%)	95% CI Lower limit	95% CI Upper limit
Apparent prevalence	32.1	32.1	23.2	41.0
True prevalence	40.6	40.6	31.2	49.9
Sensitivity	79.1	79.1	66.9	91.2
Specificity	100.0	100.0	100.0	100.0
Predictive value +	100.0	100.0	100.0	100.0
Predictive value -	87.5	87.5	79.9	95.1

Table 4. Comparison of the test evaluation of both isolation methods.

3.4. Antimicrobial resistance

As expected, all strains were resistant to cefoxitin and penicillin. More than 90% of the strains were resistant to tetracycline (96.3%) and trimethoprim (95.1%). A high prevalence of resistance was also observed to clindamycin (86.4%), erythromycin (86.4%), kanamycin (80.2%) and gentamicin (76.5%). More than half of the strains were also resistant to streptomycin (58.0%). Lower resistance levels were detected to fusidic acid (27.2%), sulfamethoxazole (25.9%), quinupristin/dalfopristin (23.5%), tiamulin (17.3%), ciprofloxacin (16.0%), rifampicin (13.6%) chloramphenicol (12.3%) and mupirocin (9.9%). No resistance were observed to linezolid and vancomycin (Table 4).

Multi-resistance was calculated and results are shown in table 6 and figure 1. It should be noted that the strains 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 9 different antibiotics. Two strains had resistance to as much as 16 different antibiotics, remaining susceptible only to ciprofloxacin, linezolid and vancomycin. The strains resistant to 15 or 16 antibiotics were all t011, they originated from 2 veal calves and 3 meat type bovines. The one strain resistant to 14 antibiotics was a t6228 strain.

The one strain resistant only to 4 antibiotics was a t1456 strain and originated from a dairy cow. The strains resistant to 4, 5 or 6 antibiotics were t011 (1 dairy cow, 2 veal calves), t1456 (meat type cow) and t1985 (veal calve and meat type cow). The MLST 30 strain was resistant

to 9 different antibiotics and the MLST 8 strain was resistant to 7 different antibiotics. Seen the low number of isolates from meat cows and dairy cows, comparison between the different groups is not relevant. However, results were similar: when there was a high prevalence of resistance in one group, this was also the case for the other groups and the same is true for the lower prevalences.

Antimicrobial	% strains at concentration														%R	Confidence interval
	≤0,0016	0,12	0,25	0,5	1	2	4	8	16	32	64	128	256	512		
CHL							4,9	46,9	35,8	2,5	8,6	1,2			12,3	6,1-22
CIP			18,5	27,2	9,9	2,5	2,5	11,1	28,4						16,0	33-56
CLI		12,3	1,2	1,2	1,2	0,0	0,0	84,0							86,4	77-93
ERY			3,7	7,4	2,5	0,0	2,5	2,5	81,5						86,4	77-93
FOX				0,0	0,0	0,0	0,0	3,7	18,5	77,8					100,0	95,5-100
FUS				72,8	12,3	2,5	9,9	2,5							27,2	17,9-38
GEN					22,2	1,2	3,7	7,4	21,0	44,4					76,5	65,8-85
KAN							16,0	3,7	2,5	2,5	9,9	65,4			80,2	69,7-88
LZD					23,5	75,3	1,2	0,0							0,0	0-4
MUP				86,4	3,7	3,7	0,0	0,0	0,0	0,0	0,0	0,0	6,2		9,9	4,4-19
PEN		0,0	0,0	0,0	1,2	8,6	90,1								100,0	95,5-100
RIF	86,4	2,5	0,0	1,2	9,9										13,6	5,2-20
SMX											70,4	3,7	11,1	14,8	25,9	16,8-37
STR							14,8	22,2	4,9	9,9	48,1				58,0	46,5-69
SYN				32,1	44,4	8,6	8,6	6,2							23,5	14,8-34
TET				2,5	1,2	0,0	0,0	0,0	1,2	95,1					96,3	86,6-99
TIA				75,3	7,4	0,0	0,0	17,3							17,3	9,8-27
TMP						4,9	3,7	1,2	0,0	1,2	88,9				95,1	87,8-99
VAN					87,7	12,3	0,0	0,0	0,0						0,0	0-4

Table 5. MICs of 81 methicillin-resistant *S.aureus* from bovines for antimicrobial tested with micro-broth dilution. Value indicated in light blue cells means that the MIC was lower than the lowest concentration tested. Values in darker blue 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
1	0	0,0	0,0
2	0	0,0	0,0
3	0	0,0	0,0
4	1	1,2	1,2
5	1	1,2	2,5
6	5	6,2	8,6
7	7	8,6	17,3
8	13	16,0	33,3
9	15	18,5	51,9
10	21	25,9	77,8
11	6	7,4	85,2
12	2	2,5	87,7
13	4	4,9	92,6
14	1	1,2	93,8
15	3	3,7	97,5
16	2	2,5	100,0
17	0	0,0	100,0
18	0	0,0	100,0
19	0	0,0	100,0

Table 6. Multi-resistance in MRSA from bovines.

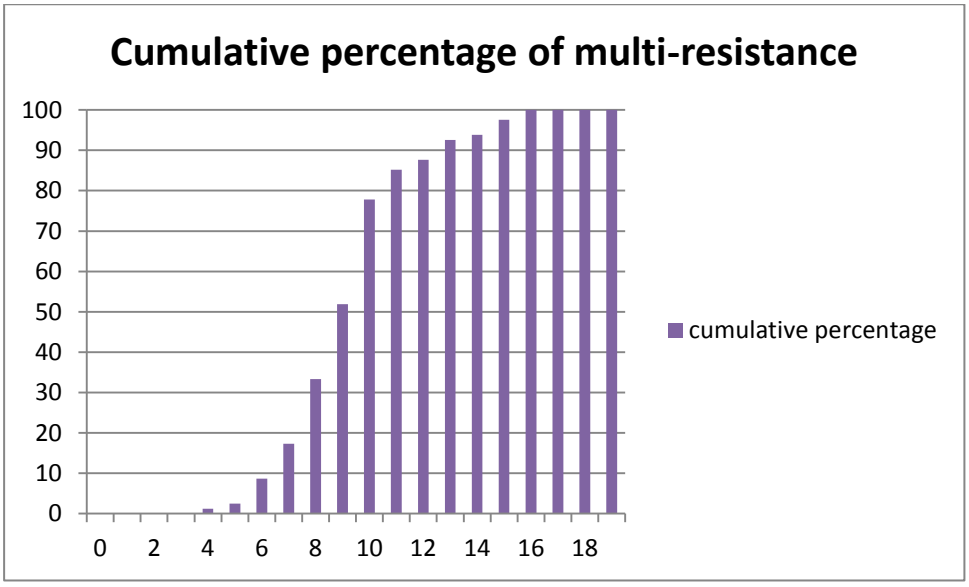

Figure 1. Cumulative percentage of multi-resistance.

3.5. MRSA characterization

Among 81 MRSA isolates recovered, seventy-eight (96.3%) were positives for the cc398 PCR and considered as MRSA ST398. Three other isolates were negative and MLST analysis performed showed one ST8 and two ST239. Ten different *spa*-types were identified. Sixty-four (79.0%) were *spa*-type t011, and others were t037, t121, t388, t1451, t1456, t1985, t3423, t6228 or non-typable (NT). MRSA *spa*-type t121 was associated to MLST type ST8, t388 and t037 to ST239. Forty-six (56.8%) isolates carried SCC*mec* type IV (2B) and eleven (13.6%) SCC*mec* type IV (2B&5). Sixteen (19.8%) isolates carried SCCmec type V (5C2) and two (2.5%) SCC*mec* type III (3A). Six (7.4%) isolates were non typable (NT) using Kondo's method (Table 6).

	MLST			*spa*-types										SCC*mec*				
	8	239	398	t011	t037	t121	t388	t1451	t1456	t1985	t3423	t6228	NT	III (3A)	IV (2B)	IV (2B&5)	V (5C2)	NT
Dairy farms	0	2	12	8	1	0	1	0	1	0	0	2	1	1	4	0	6	3
Beef farms	1	0	18	16	0	1	0	0	1	1	0	0	0	1	12	0	3	3
Veal farms	0	0	48	40	0	0	0	3	1	3	1	0	0	0	30	11	7	0
Total	1	2	78	64	1	1	1	3	3	4	1	2	1	2	46	11	16	6

Table 6. Total number of MRSA isolates corresponding to the different genotypes recovered and separated by farm types.

4. Discussion and conclusion

The overall MRSA prevalence in bovines was 19.7%. This is much higher than the prevalence observed in poultry in 2011, but lower than the surveillance in pigs in 2007 and 2009. It should be noted however that for the surveillance in pigs, the methodology was quite different. As for the 2007 surveillance, 30 individual samples were taken per farm, and as for the 2009 surveillance (organised by EFSA), pooled dust samples were used. It was shown that the last method was not as sensitive as animal testing. In this study, 20 nasal samples were taken and pooled for analysis at the laboratory. Pooling of samples is interesting, though might have a lowered sensitivity when at farm level, prevalence is low. There are however few data available on the prevalence of MRSA in the nose of cows at farm level (Vandendriessche et al., 2013).
A first study in Belgium gave some indication of the high prevalence of MRSA in veal calves (Vandendriessche et al., 2013). In the Netherlands the high prevalence of resistance in veal calves was clearly demonstrated (Graveland et al, 2010). Here 88% of the farms were proven positive. The lower prevalence in our study may be explained by the differences in sampling. In the Dutch study, a more sensitive sampling was applied. Ten to 25 individual samples per farm were analysed. Lower prevalences were found in dairy cattle. Before in Belgium, MRSA CC398 was shown to be implicated in mastitis (Vanderhaegen et al., 2010) and in the nose of dairy cattle, however the latter study implicated only few farms (Vandendriessche et al., 2013). In this study, a low prevalence was found (1%). Today, the prevalence is at nearly 10%. However, care should be taken since in the actual study, a less sensitive method was used, but a larger sample. As such one cannot state that there is a difference with the former study. The presence of MRSA CC398 in dairy cattle is however of extreme importance seen that they may cause mastitis (Vanderhaegen et al., 2010). On positive farms, measures should be taken to eliminate as much as possible these MRSA strains to avoid they cause mastitis. The prevalence in beef cattle is the same as for dairy cattle. It would be interesting to investigate possible age related prevalence, as was seen in pigs (Crombé et al., 2012).
Also in this study we compared different isolation methods. In the study in a low prevalence population (poultry), we have demonstrated that there was no improvement in isolation rate using 2 different enrichments (Némegaire et al., accepted). In this study, in a population with a higher MRSA prevalence there was also no significant difference between the two methods. This indicates that the second enrichment method is not adding any value to the obtained results and may be omitted.

Most isolates were resistant to tetracycline, trimethoprim. In this study two CC398 isolates were found to be susceptible to tetracycline, tetracycline susceptible strains are only very rarely found. Also trimethoprim susceptible strains are rarely found. It has been shown that the gene in these strains is still present but not functional anymore (Verhegghe et al., unpublished results). The prevalence of erythromycin, clindamycin, kanamycin and gentamicin in this collection is extremely high compared to what has been found in strains from other origins in Belgium (mainly pig strains). The level of multi-resistance is extremely high. On 19 antimicrobials tested, no one was susceptible to

all, but that is of course due to the fact that these are already selected for methicillin resistance, rendering them automatically resistant to the two β-lactam antibiotics tested. Nevertheless the strains with the lowest level of multi-resistance were resistant to 2-3 additional antibiotics (and this was only 2 strains). Two isolates were resistant to sixteen antimicrobials out of nineteen tested excluding ciprofloxacin, linezolid and vancomycin, three antibiotics that are last resort antibiotics in the treatment of MRSA infections in humans. If these strains would spread in mastitis in bovines, these animals would not be treatable anymore.

Most isolates were typical LA-MRSA ST398, *spa*-type t011. Other less prevalent *spa*-types associated to ST398 were also recovered. However, MRSA ST8 *spa*-type121 and MRSA ST239 *spa*-type t037 and t388 are classified among hospital-acquired (HA)-MRSA. MRSA *spa*-type t121 was previously recovered in a Belgian hospital in 2002 and 2007 (Wildemauwe *et al.* 2009) and is commonly found in Europe and in the United States (**http://spa.ridom.de**). This *spa*-type has also been found in bulk tank milk in the United States (Haran et al. 2011). MRSA ST239 *spa*-type t388 and t037 are widespread HA-MRSA found in Europe, Asia, and America (Campanile *et al.* 2010[8]). MRSA ST239 t037 was recovered also among MRSA in poultry during the previous Belgian survey in poultry in 2011. This interesting finding confirms the possible spread of HA-MRSA to livestock hypothesized in 2011. This situation should also be followed up closely since it may be an indication of a new animal adapted MRSA strain originating from humans. Also CC398 strains originated from humans and adapted to animals (Price et al., 2012).

The diversity of *spa*-types in bovines was larger than what has been found in pigs during surveys in 2007 (Crombé et al, 2012) and during the EFSA baseline surveillance in pigs in 2009 (EFSA report, **http://www.efsa.europa.eu/en/efsajournal/pub/1597.htm**). In both studies, only spa types t011 and t034 were found. Here at least 7 CC398 spa-types were found. The *spa* gene is a surface protein that is under the selective pressure of host immunity. Differences in this immunity might be the indication that MRSA CC398 is changing its profile according to the host and that some host adaptations are underway. In 2013, a new surveillance in pigs is organised and it will be interesting to see whether the diversity has changed also in pigs.

As expected, SCC*mec* type IV was the most common type in veal Calves (Cavaco *et al.* 2010) followed by SCC*mec* type V. In pigs, this was the other way round, being type V as the most prevalent cassette. Two isolates were carrying SCC*mec* type III(3A) and six isolates carried a non-typable SCC*mec* cassette. The type III(3A) SCCmec found here is not the classical type III found in HA-MRSA. It might also be an aberrant type V.

In conclusion, MRSA has been found at lower prevalences in the nares of dairy and meat cows, but at elevated levels in veal calves. The diversity of strains was larger than what was seen in pigs, including also some more typically hospital associated MRSA. The diversity in SCCmec cassettes in CC398 is not

anymore limited to the types IV and V but includes now also the type III cassette. Converse to pigs, the most prevalent SCCmec type was IV while in pigs it was type V, associated with zinc resistance.

5. References

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