CODA

CENTRUM VOOR ONDERZOEK IN DIERGENEESKUNDE EN AGROCHEMIE

MRSA surveillance 2011: poultry

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1. Introduction

The prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in pigs is well recognized. However, little is known about the prevalence in other animal species. In the framework of the FASFC surveillance and the EMIDA-ERA NET project, a surveillance of MRSA in poultry has been executed. For this surveillance, the by 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. While for pigs, dust from the barn was used, pooled nasal samples were used in the poultry surveillance.

We compared two different isolation methods, the method advised by EFSA and an alternative method applied in the Veterinary and Agrochemical Research Centre (VAR) on pooled nasal swabs from broilers and laying chickens.

2. Material and methods

2.1. Sample origin

On each of the 372 farms, 20 chickens have been sampled during 2011. Ninety-two farms raised broilers chickens and 280 farms were egg producing farms.

2.2. Isolation methods

2.2.1. Isolation method EFSA

For all of the 372 farms, samples were pooled per farm and incubated in Mueller-Hinton (MH) broth 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 colonise were purified on a Columbia Sheep Blood (CSB) agar plate and incubated overnight at 37°C.

2.2.2. Adapted Isolation method VAR

The alternative protocol was applied to 334 farms and was very similar to the EFSA 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 [1]. 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 nuc1 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[2]. This method allows the rapid detection of the *S. aureus* sequence type (ST)398.

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 al, 2000[3]. 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 Cohen's kappa coefficient (2x2 table) was calculated in order to compare the two isolation methods. Cohen's kappa coefficient was interpreted as shown in the table 1 (Landis and Koch, 1977[4]). This analysis was done on those 334 farms for which both isolation methods (outcome Positive/Negative) were available. EFSA method is considered as the Gold Standard while a Var isolation method 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 theCohen's kappa coefficient

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio positive (LR+) and negative (LR-) of both methods (Table 2) were also calculated using the following formulae (Dohoo et al.,2009)[5]:

Sensitivity= $\frac{\#True\ positives\ (TP)}{\#\ True\ positives\ (TP) + False\ negatives\ (FN)}$

Specificity= $\frac{\#\ True\ negatives\ (TN)}{\#\ True\ negatives\ (TN) + False\ positives\ (FP)}$

PPV= $\frac{\#\ True\ positives\ (TP)}{\#\ True\ positives\ (TP) + False\ positives\ (FP)}$

NPV= $\frac{\#\ True\ negatives\ (TN)}{\#\ True\ negatives\ (TN) + False\ negatives\ (FN)}$

LR+ = Se / 1-Sp

LR- = 1 - Se / Sp

		Condition (determined by the Gold Standard)	
		Positive	Negative
Test outcome	Positive	TP	FP
	Negative	FN	TN

Table 2. Calculation of sensitivity, specificity, PPV and NPV of the isolation method.

Cohen's Kappa, Pearson chi square and Fisher's exact test have been computed using IMB SPSS Statistics® Version 20.0.

3. Results

3.1. MRSA detection and identification

3.1.1. Prevalence EFSA

Over 334 farms tested in parallel, only three were positive for MRSA with the EFSA isolation method. All these positive farms raised broiler chickens leading to a total prevalence of 3% for broiler farms (table 3).

		EFSA		Total
		Positive	Negative	
VAR	Positive	1	5	6
	Negative	2	326	328
Total		3	331	334

Table 3. Results for EFSA and VAR isolation methods.

3.1.2. Prevalence VAR

Six farms were positive with the VAR method. Positives farms from EFSA methods were all raising broilers. Two positive farms from VAR methods were raising layers and four were raising broilers (table 4).

3.1.3.Comparison EFSA-VAR

Only one farm was found positive by both methods. Two farms detected positive with the EFSA have not been detected with the VAR method. Five farms detected positive with the VAR have not been detected with the EFSA method. A total of eight farms were then found to be MRSA positive using both isolation methods in parallel (table 5).

Isolation method	Broilers	Layers
EFSA	3.3 (n=92)	0.0 (n=280)
VAR	4.8 (n=81)	0.8 (n=251)

Table 4. MRSA prevalence in the different farms. Prevalence in % for EFSA method is computed out of 92 broilers and 280 layers while computed out of 81 broilers and 251 layers while out of 334 for VAR method.

Isolation method	N farms positive	Prevalence in % (number of samples tested)	Farm type
EFSA	3	0.8 (n=372)	Broilers
VAR	6	1.8 (n=334)	2 Layers and 4 broilers
EFSA & VAR	8	2.4	Layers and broilers

Table 5. Number of MRSA isolated with both isolation methods. Prevalence for EFSA method is computed out of 372 while out of 334 for VAR method. EFSA & Var is the comparison of both methods used in parallel.

Cohen's kappa coefficient k = 0.21 which means that there is a fair agreement between these two methods. The p value for the Fisher's exact test was higher than 0.05 (χ^2= 1.392; df=1; p=0.319) indicating then that there is no significant difference between both methods. Based on the assumption that all farms that tested positive in at least one test are true positive farms (n=8), the sensitivity of EFSA isolation method and of the adapted VAR method is 0.375 (=3/8) and 0.75 (= 6/8) respectively. Both PPV's are equal to 1 since there are no false positives. NPV of the EFSA and VAR methods are 0.985 (= 326/[326+5]) and 0.994 (=326/[326+2]) respectively. The LR+ for EFSA and VAR methods was 25 and 125 respectively, whereas the LR- for EFSA and VAR methods was 0.635 and 0.252 respectively.

Specificity, positive predictive value (PPV) and negative predictive value (NPV) for both methods were not significantly different ($p>0.05$; table 6). However, the p value for the Fisher's exact test comparing the prevalence in broilers and layers shows a significant difference for these two groups ($\chi^2= 13.923$; df = 1; p= 0.001). Finally, there is no significant difference in the number of broilers and layers in both methods ($\chi^2= 0.11$; df= 1; p= 0.930).

	EFSA	VAR
Sensitivity	0.375	0.75
Specificity	1	1
PPV	1	1
NPV	0.985	0.994

Table 6. Comparison of the sensitivity, specificity, the Positive Predictive Value and the Negative Predictive value

The two MRSA that have not been detected in the VAR method were considered as False Negative in the calculation of the sensitivity and of the NPV for this method. In the EFSA method, five MRSA have not been detected and were then also considered as False Negative. Therefore the NPV EFSA is lower than NPV VAR (but non-significantly), on the assumption that none of the other farms were false negatives due to not being detected by any of the two tests.

3.2. Genotyping

The four CC398 strains belonged to spa-type t011 and one to type t899 (table 7). The MLST type 398 has been reported as typical Livestock Associated (LA-)MRSA. The three other strains belong to the spa-type t037 which was not positive for the CC398. The MLST revealed that these strains were ST239. This type has been reported as Hospital Acquired (HA-)MRSA. Although this sequence type is considered as worldwide and one of the oldest pandemic MRSA including in Europe, spa-type t037 has not been described in Belgium yet to our knowledge.

spa-type	cc398 PCR	MLST	Number of strains	Origin
t011	Positive	ST398	4	Broilers
t037	Negative	ST239	3	Two broilers, one layers
t899	Positive	ST398	1	Broilers

Table 7. Results of the spa-type, CC398 PCR and MLST applied the eight MRSA strains.

3.3. Antimicrobial resistance

Susceptibilities are shown in table 8. All strains were evidently resistant to cefoxitin and penicillin. All strains were also resistant to erythromycin and typically for ST398 to tetracycline. No strain was resistant to linezolid, mupirocin and vancomycin. ST239 strains showed all the same resistance profile and were all resistant to chloramphenicol, kanamycin, rifampicin, sulfamethoxazole and streptomycin. None were resistant to ciprofloxacin, clindamycin, fusidic acid, gentamycin, quinupristin/dalfopristin, tiamulin, and trimethoprim. All ST398 strains were resistant to clindamycin and trimethoprim. Four were resistant to gentamicin and kanamycin and four were resistant to ciprofloxacin. Two ST398 strains were resistant to sulfamethoxazole and streptomycin. Two were resistant to chloramphenicol, fusidic acid or tiamulin. One ST398 strain was resistant to rifampicin and one to quinupristin/ dalfopristin.

Strains	Spa-type	CHL	CIP	CLI	ERY	FOX	FUS	GEN	KAN	LZD	MUP	PEN	RIF	SMX	STR	SYN	TET	TIA	TMP	VAN
72	**t037**	64	<=0.25	<=0.12	>8	>16	<=0.5	<=1	>64	2	<=0.5	>2	>0.5	512	>32	<=0.5	>16	<=0.5	<=2	<=1
86	**t037**	64	<=0.25	<=0.12	>8	>16	<=0.5	<=1	>64	<=1	1	>2	>0.5	512	>32	<=0.5	>16	<=0.5	<=2	<=1
282	**t037**	64	<=0.25	<=0.12	>8	>16	<=0.5	<=1	>64	<=1	<=0.5	>2	>0.5	>512	>32	<=0.5	>16	<=0.5	<=2	<=1
118	t899	<=4	2	>4	4	16	1	8	64	2	<=0.5	>2	0.03	<=64	8	1	>16	>4	>32	<=1
213	t011	64	0.5	>4	>8	>16	2	>16	>64	<=1	<=0.5	>2	>0.5	256	>32	<=0.5	>16	1	>32	<=1
286	t011	16	2	>4	>8	16	<=0.5	<=1	<=4	2	<=0.5	>2	<=0.016	<=64	8	1	>16	<=0.5	>32	<=1
363	t011	32	2	>4	>8	16	<=0.5	>16	>64	<=1	<=0.5	>2	<=0.016	256	>32	4	>16	>4	>32	<=1
371	t011	8	>8	>4	>8	16	<=0.5	>16	64	2	<=0.5	>2	<=0.016	<=64	8	1	>16	2	>32	<=1

Table 8. Results of the antimicrobial resistance for all strains isolated. Yellow= susceptible, red: resistant. Type ST239 are in bold

4. Conclusion and discussion

This surveillance confirms the presence of MRSA in poultry (Nemati et al. 2009[6], Persoons et al. 2009[7]) however the prevalence in chickens is low; between 0.8% to 2% depending on the isolation methods. Prevalence per farm type shows that the prevalence is higher in broilers than in layers. Since 75% of sampled farms were egg producing, this could explain the low total prevalence. The comparison between both isolation methods shows that use of TSB supplemented with cefoxitin in this population may be too selective for the detection of MRSA. Indeed, only three farms were detected as positive with the EFSA methods while VAR method detected six MRSA. Although the differences between the two methods are not statistically significant, the most sensitive isolation method is preferred, since it is important to detect as many positive farms as possible in a low prevalence environment to avoid the further spread to other farms.

Over these eight MRSA, five belong to the livestock associated ST398 *spa* type t899 and t011. The three other MRSA belong to the HA-MRSA ST239 *spa* type t037. This sequence type is considered as one of the oldest pandemic MRSA with an ancestral lineage in Europe. It account for 90% of the HA-MRSA in Asia and has been detected in South America and also recently circulating in Eastern Europe. This sequence type shows geographic variations in terms of the *spa* type. spa-type t037 has been recently reported in different country in Asia and in Russia. However according to the comparison of the phylogeny, t037 represents the ancestral ST239 *spa* type (Harris et al. 2010[8]), this is, at our knowledge, the first report of *spa* type t037 in Belgium.

Concerning antimicrobial resistance, all strains were resistant to at least seven antimicrobials and to maximum fourteen out of nineteen antimicrobials tested. As expected, all strains were resistant to penicillin and to cefoxitin and also to erythromycin and tetracyclin. None was resistant to linezolid, mupirocin and vancomycin. The three ST239 showed the same resistance pattern while ST398 showed different pattern of resistance.

5. References

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