

## THE ISOLATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AT BREEDING PIG FACILITIES IN CROATIA

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### Summary

Methicillin resistant *Staphylococcus aureus* (MRSA) have emerged worldwide and have become resistant to a variety of antibiotics. MRSA colonisation in pigs was first reported in the Netherlands at 2005, where pigs were implicated as a source of human MRSA infections. Dust samples were collected from six large pig farms in Croatia from breeding pig facilities. On each farm, between 5 and 6 samples were taken by swabs. Of the total 32 swabs, isolates from 8 swabs from four of the six examined farms grew pink colonies on the MRSA select agar. The *mecA* gene was detected in all 8 isolates growing pink colonies on the MRSA agar. All isolates are resistant to penicillin, ampicillin, oxacillin, oxitetracycline and streptomycin. All isolates were susceptible to vancomycin, ciprofloxacin, florfenicol and sulfametoxazole/trimethoprim.

**Keywords:** MRSA; pigs; facilities

### INTRODUCTION

*Staphylococcus (S.) aureus* causes severe animal diseases, such as suppurative diseases, mastitis, arthritis and urinary tract infections with numerous virulence factors. For humans, this organism is a common cause of skin and soft tissue infection [1].

Human isolates of *S. aureus*, unlike animal isolates, are frequently resistant to the penicillinase-resistant penicillins [2]. An organism exhibiting this type of resistance is referred as methicillin (oxacillin) - resistant *S. aureus* (MRSA). MRSA strains are resistant to all beta-lactam antimicrobials through a penicillin binding protein (PB-P2a) that has a low affinity for all beta-lactams. This is encoded by the *mecA* gene,

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which resides on a mobile genetic element called a staphylococcal chromosomal cassette (SCCmec). Additionally, MRSA strains are often resistant to a wide range of commonly used antimicrobials, including aminoglycosides, macrolides, chloramphenicol, tetracycline and fluoroquinolones [3,4].

Swine have recently emerged as another reservoir of *S. aureus*, including MRSA. The majority of swine-associated *S. aureus* belong to the same multi locus sequence type (MLST) ST398. This sequence type has also been referred to non-typeable MRSA due to the inability of the *Sma*I restriction enzyme to cut DNA from these strains due to the presence of a unique methylase [5].

## MATERIALS AND METHODS

### Sample collection

Dust samples were collected from six large pig farms in Croatia. The number of selected samples and the number of positive samples by farms are presented in Table 1.

On each farm, between 5-6 dust samples were taken from the facilities with breeding animals. A total of 32 swabs were taken. Each swab was used to sample approximately 500 cm<sup>2</sup> surface area with dust, after which swabs were stored in sterile plastic test tubes. The method of sampling is standard procedure described elsewhere [6].

### Isolation of MRSA

At the laboratory, each swab was grafted in 100 mL Mueller-Hinton broth supplemented with 6.5 % NaCl and incubated at 37 +/- 1°C for 16–20 h. One loop-full was spread over chromogenic agar selective for MRSA (MRSA Select™ agar, Biorad 63747). Based on the stain, a subculture was taken from each MRSA agar growing pink colonies and transferred to blood agar, where growth morphology and haemolysis were controlled (double haemolysis zone). Such strains were kept on semi-soft agar (Stock culture, Biorad 63683) at 2–8° until further research.

### Determination of MIC of oxacillin

The minimum inhibitory concentration (MIC) of oxacillin was determined by the E-test (AB BioMérieux, Sweden). The test is an expansion of the disc diffusion method with the same agar and inoculum preparation. The antibiotic content of the strip is graded, and the concentration is printed linearly along the strip. The test was performed according to manufacturer's instructions.

Oxacillin concentrations ranged from 0.016 to 256 µg/ml. Mueller-Hinton agar with 2% NaCl was used as culture medium (Merck 1.05435), while *S. aureus* ATCC 25923 were used as the control strain. Plates were incubated at 35°C for 24 hours and examined under transmitted light.

### **Amplification of the *mecA* gene**

The presence of the *mecA* gene was verified for oxacillin resistant isolates by means of PCR described elsewhere [7].

*S. aureus* colonies were suspended in 50 µl of Q water (Sigma, Germany), heated to 99°C for 20 minutes and centrifuged at 14 000 g for 1 minute. The supernatant was used for the PCR as the DNA template.

Primers used to confirm the presence of the *mecA* gene were MecA-1 (GGG ATC ATA GCG TCA TTA TTC) and MecA-2 (AAC GAT TGT GAC ACG ATA GCC). The product of the amplification using these two primers was 527 base pairs (bp) long. Primers NUC-1 (TCA GCA AAT GCA TCA CAA ACA G) and NUC-2 (CGT AAA TGC ACT TGC TTC AGG) were used to confirm the presence of nuclease specific for *S. aureus*. The size of the amplified nuclease fragment was 255 bp. The presence of DNA was confirmed by primers 16S-1 (GTG CCA GCA GCC GCG GTA A) and 16S-2 (AGA CCC GGG AAC GTA TTC AC) which gave an 886 bp long amplification product.

CR was carried out in a 50 µl reaction mixture containing 25 µl of Multiplex PCR Master Mix, 5 µl of Q-Solution, 12.5 µl water (Qiagen, Germany), 1.3 µM of each of the primers (Invitrogen, Scotland) and 5 µl of DNA. Thermal cycling was accomplished with a GeneAmp® PCR System 2700 (Applied Biosystems, USA). After initial denaturation (95°C/15 min), the PCR profile was as follows: 35 cycles of denaturation (94°C/30 sec), annealing (55°C/30 sec) and extension (72°C/1 min), with a final extension step (72°C /10 min).

Amplification products were separated in a 1.5% agarose gel and stained by ethidium bromide. Visualization was carried out by the UV transluminator and the camera BioCapt Document System (Vilbert Lourmat, France).

### **Antimicrobial susceptibility testing**

The susceptibility of all *mecA* positive isolates to different antimicrobial agents was tested using the disc diffusion method as standardised by CLSI [8].

Susceptibility testing was conducted using the disk diffusion method according to [8] for the following antimicrobial agents: penicillin G (10 IU), ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), erythromycin (15 µg), cefotaxime (30 µg), ceftriaxone (30 µg), oxytetracycline (30 µg), streptomycin (10 µg), neomycin (30 µg),

gentamicin (10 µg), ciprofloxacin (5 µg), florfenicol (30 µg) and trimethoprim + sulfamethoxazole (1.25 + 23.75 µg).

According to CSLI, Mueller-Hinton agar was used as a culture medium (Merck 1.05435). *Staphylococcus aureus* ATCC 25923 was used as the control strain.

Plates were incubated at 35°C for 24 hours and examined under transmitted light. The zone of growth inhibition was interpreted as sensitive, moderately sensitive and resistant, as recommended by CSLI [8].

## RESULTS

### Isolation of MRSA from swabs

Of the total 32 swabs, isolates from 8 swabs from 4 of the 6 examined farms grew pink colonies on the MRSA select agar and had a double haemolysis zone on blood agar (Table 1).

**Table 1.** Number of positive samples and total number of dust samples on farms

Farm No	Positive samples / Total number of samples taken from facilities with breeding animals
1	4/6
2	0/5
3	1/5
4	0/5
5	2/5
6	1/6
Total	8/32

### Detection of *mecA* gene

The *mecA* gene was detected in all 8 isolates growing pink colonies on the MRSA agar. *S. pseudintermedius* isolates were used as controls. In *S. pseudintermedius*, the *mecA* gene and gene specific for *S. aureus* nuclease were not detected.

### Antimicrobial susceptibility testing

Results of the determination of antimicrobial susceptibility to 15 antimicrobial products are presented in Table 2, where specific susceptibility for each studied strain is presented.

**Table 2.** Susceptibility of MRSA isolates with disc diffusion methods and MIC to oxacilin

Isolate <sup>a</sup>	Susceptibility <sup>b</sup>															Mic Ox <sup>c</sup>
	PG	AM	OX	E	VA	AMC	CIP	OTC	S	N	GM	CTX	CRO/CAZ	FFC	SXT	
1/1	R	R	R	S	S	I	S	R	R	S	S	I	I	S	S	8
1/3	R	R	R	S	S	S	S	R	R	S	S	R	R	S	S	6
1/4	R	R	R	S	S	I	S	R	R	S	S	R	R	S	S	6
1/5	R	R	R	S	S	I	S	R	R	S	S	I	R	S	S	8
3/3	R	R	R	S	S	S	S	R	R	S	S	I	R	S	S	4
5/3	R	R	R	R	S	S	S	R	R	I	I	I	R	S	S	8
5/5	R	R	R	R	S	S	S	R	R	S	S	I	R	S	S	6
6/1	R	R	R	R	S	S	S	R	R	S	S	I	R	S	S	8

## DISCUSSION

This is the study of the presence of MRSA in large breeding pig facilities in Croatia. The study included taking dust swabs from facilities of six large pig farms in Croatia. Of the six studied farms, positive swabs were found at four farms.

The first farm at which MRSA was not isolated is a farm with obsolete technology and without any investments in technological development in recent years, while the other farm is completely new (built three years ago with modern technology and purchase of high quality breeding animals). As such, the MRSA result on the farms cannot be related with farm technology.

On other farms, there were 1 to 4 positive swabs. MRSA was isolated from 8 of the total 32 swabs (25 %).

All 8 strains which grew pink colonies on MRSA agar were tested for the presence of the *mecA* gene by PCR and provided a positive reaction. Their MIC to oxacillin was determined, and ranged between 3 and 12 g/L (Table 2).

The oxacillin MIC was relatively low (MIC<sub>50</sub> 8 mg/L, MIC<sub>90</sub> 12 mg/L, MIC range 4-8 mg/L). Further research should be supplemented with type determination of isolated MRSA isolates with *spa*-typing and MLST.

The disc diffusion method was used to determine that all isolates are resistant to penicillin, ampicilin, oxacillin, oxitetracycline and streptomycin. Total resistance to penicillin and ampicilin is an expected result, as it has been previously described [4,9].

Total resistance to oxytetracycline and streptomycin could be expected for the long-term use of such products at large pig farms in Croatia. A similar result of re-

sistance to tetracycline was obtained by de Neeleng et al. [9] in case where isolates originated from farms with a long history of use of tetracycline antibiotics.

Isolates from two farms were susceptible to erythromycin. All isolates were susceptible to vancomycin, ciprofloxacin, florfenicol and sulfametoxazole with trimetoprim which is in compliance with the results of earlier studies [9].

In recent years, there has been increasing evidence that pigs can be a source of MRSA for humans [1,10], which is supported by the fact that MRSA isolation could be much more frequent among persons having contact with pigs than among persons outside hospitals [9,11]. Persons at risks include pig farmers, transporters, personnel of slaughterhouse and veterinarians [9]. A higher prevalence of *S. aureus* carriage among pig farmers was noted in France [12]. The results presented here show that MRSA is present on a facilities of breeding pigs at large farms in Croatia and that the research should be continued with further type determination of strains, tests on animals and people in contact with pigs, to obtain a better picture of the incidence of MRSA infection in pigs and humans.

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### Sažetak

#### **Izdvajanje meticilin-rezistentnih sojeva bakterije *Staphylococcus aureus* u objektima s rasplodnim svinjama u Hrvatskoj**

Meticilin-rezistentni sojevi bakterije *Staphylococcus aureus* (MRSA) izdvajaju se u cijelom svijetu i otporni su na različite antimikrobne lijekove. Infekcije svinja sojevima MRSA-e prvi su put opisane u Nizozemskoj 2005. s pretpostavkom da su svinje izvor infekcije MRSA-om za ljude. Uzorke prašine prikupljali smo sa šest velikih svinjogojskih farmi u Hrvatskoj iz objekata u kojima su smještene rasplodne životinje. Na svakoj farmi uzeli smo pet ili šest brisova. Od ukupno uzorkovana 32 brisa, iz osam brisova s četiri farme izdvojili smo ružičaste kolonije na MRSA selektivnom agaru. Kod svih izolata koji su rasli na MRSA selektivnom agaru kao ružičaste kolonije izdvojen je gen *mecA*. Svi izolati bili su otporni na penicilin, ampicilin, oksacilin, oksitetraciklin i streptomycin, a osjetljivi na vankomicin, ciprofloksacin, florfenikol i sulfametoksazol s trimetoprimom.

**Ključne riječi:** MRSA; svinje; objekti