THE PRESENCE OF METHICILLIN-RESISTANT
STAPHYLOCOCCUS AUREUS ON LARGE PIG BREEDING
FARMS IN CROATIA

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Methicillin-resistant Staphylococcus aureus (MRSA) have emerged worldwide and have become resistant to a variety of antibiotics. MRSA colonisation in pigs was first reported from the Netherlands in 2005, where pigs were implicated as a source of human MRSA infections (Voss et al., 2005). This paper presents the first report on the presence of MRSA on large pig breeding farms in Croatia, together with the determination of the mecA gene, the results of spa typing and susceptibility to commonly used antimicrobials. Dust samples (7–11 per farm) were collected from eight large pig farms in Croatia. Of the total 68 swabs, the mecA gene was detected in 24 isolates growing on the MRSA agar. All isolates were resistant to oxacillin, tetracycline and streptomycin, and susceptible only to vancomycin, while 92% of the strains were susceptible to ciprofloxacin. Genotyping of the MRSA strains was performed by spa typing, and revealed t011 (n = 17), t034 (n = 5) and t1451 (n = 2). The results presented here predict that MRSA is present on a large number of pig farms in Croatia.

Key words: Methicillin-resistant Staphylococcus aureus, pigs, antimicrobial susceptibility

Staphylococcus (S.) aureus causes severe animal diseases, such as suppurative processes, mastitis, arthritis and urinary tract infections with its numerous virulence factors. For humans, this organism is an important cause of food poisoning, pneumonia, postoperative infection and nosocomial infection (Lee, 2003).

Methicillin-resistant Staphylococcus aureus (MRSA) is usually a multidrug resistant Gram-positive bacterium (Khanna et al., 2008). An organism exhibiting this type of resistance is referred to as methicillin- (oxacillin-) resistant S. aureus through a penicillin-binding protein (PBP2a) that has a low affinity to all beta-lactams. This is encoded by the mecA gene, which resides on a mobile genetic element called a staphylococcal cassette chromosome mec (SCCmec).

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Additionally, MRSA strains are often resistant to a wide range of commonly used antimicrobial agents, including aminoglycosides, macrolides, phenicols, tetracyclines and fluoroquinolones (Lee, 2003; Khanna et al., 2008).

MRSA strains have emerged worldwide and the prevalence in humans varies widely between countries, from less than 1% in the Netherlands (Voss et al., 2005) to more than 20% in other European countries such as Belgium, Ireland and the United Kingdom (Tiemersma et al., 2004).

MRSA colonisation in pigs was first reported in the Netherlands where pigs were implicated as a source of human MRSA infections. Until now, MRSA has been identified in pigs in France, the Netherlands, Denmark, Singapore, Japan and several other countries (Khanna et al., 2008; Baba et al., 2010).

The aim of this study was to determine, for the first time, the presence of MRSA on large pig breeding farms in Croatia, and to report the results of mecA gene determination, spa typing and susceptibility to commonly used antimicrobials in pig farms and the minimum inhibitory concentration (MIC) of oxacillin.

**Materials and methods**

**Sample collection**

Dust samples were collected from eight large pig farms (with 470 to 3200 sows and a corresponding number of boars for artificial insemination) in Croatia. These farms have a total of 9000 sows, or 40% of the sows under selection in Croatia. The number of selected samples and the number and percentage of positive samples by farm are presented in Table 1.

<table>
<thead>
<tr>
<th>Herd no.</th>
<th>Herd type</th>
<th>Samples</th>
<th>Spa type (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>total (n)</td>
<td>positive (n)</td>
</tr>
<tr>
<td>9280</td>
<td>farrow-to-finish</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>10454</td>
<td>farrow-to-finish</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>21175</td>
<td>farrow-to-finish</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>22533</td>
<td>farrow-to-finish</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>31955</td>
<td>farrow-to-finish</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>33141</td>
<td>farrow-to-finish</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>37122</td>
<td>breeding</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>37126</td>
<td>breeding</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

Seven to 11 samples were taken by dry sterile swabs on each farm. Five to six dust samples were taken from the breeding herds and 7–11 samples from the farrow-to-finish herds. A total of 68 swabs were taken. Each swab was used to
sample dust from a surface area of approximately 500 cm², and then the swabs were stored in sterile plastic tubes.

**Isolation of MRSA**

Swabs were inoculated into 100 mL Mueller-Hinton broth supplemented with 6.5% NaCl and incubated at 37 ± 1 °C for 16–20 h. One loopful was inoculated onto chromogenic agar selective for MRSA (MRSA Select™ agar, Biorad 63747). A subculture was taken from each MRSA agar growing pink colonies and transferred to blood agar, where colony morphology and haemolysis were controlled. All MRSA strains were tested for the presence of the **mecA** gene by multiplex PCR.

**Multiplex PCR for the identification of MRSA**

The presence of the **mecA** gene was tested by PCR as described elsewhere (Anonymous, 2008). Primers used for confirming the presence of the **mecA** gene were MecA-1 and MecA-2. Primers NUC-1 and NUC-2 were used to confirm the presence of nuclease specific for *S. aureus*. The presence of DNA was confirmed by primers 16S-1 and 16S-2. Susceptible *S. pseudointermedius* strain was used as control.

**Spa typing**

*Spa* typing was performed as described (Shopsin et al., 1999; Aires-de-Sousa et al., 2006). PCR products were purified using the Exosap (USB, Staufen, Germany) and sequenced in both directions (Macrogen, Seoul, Korea). Ridom database equivalents were identified using the Ridom Spaserver website (www.spaserver.ridom.de).

**Antimicrobial susceptibility testing**

The antibiotic susceptibility of all **mecA** positive isolates was tested using the disc diffusion method according to the Clinical and Laboratory Standards Institute (2008). The isolates were tested with a panel of 9 antibiotics: erythromycin (15 µg), vancomycin (30 µg), tetracycline (30 µg), streptomycin (10 µg), neomycin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), florfenicol (30 µg) and trimethoprim + sulphamethoxazole (1.25 + 23.75 µg). Plates were incubated at 35 °C for 24 h and examined under transmitted light. The zone of growth inhibition was interpreted as sensitive or susceptible, intermediate and resistant, as recommended by the CLSI M31 A3 document (Clinical and Laboratory Standards Institute, 2008).

The MICs of oxacillin were determined by the E-test (AB BioMérieux, Sweden). Oxacillin concentrations ranged from 0.016 to 256 µg/ml. Mueller-Hinton agar with 2% NaCl was used as culture medium. *Staphylococcus aureus* ATCC 25923 served for quality control in both methods.
Results

Isolation of MRSA from swabs

Of the total 68 swabs, isolates from 24 swabs from six of the eight examined farms grew pink colonies on the MRSA select agar and had a double haemolysis zone on blood agar (Table 1). On positive farms, there were 1 to 9 positive swabs. MRSA was isolated from 24 of the total 68 swabs (35%).

Detection of the mecA gene

All of the 24 strains which grew pink colonies on MRSA agar were positive for the presence of the mecA gene by PCR. The S. pseudointermedius strain was used as control. This S. pseudointermedius strain was susceptible to oxacillin, amoxicillin + clavulanic acid, cefotaxim and ceftriaxone, and had an oxacillin MIC of ≤ 2 mg/L. The mecA gene and the gene specific for S. aureus nuclease were not detected in S. pseudointermedius.

Antimicrobial susceptibility testing

The results of the determination of antimicrobial susceptibility to 9 antimicrobials are presented in Table 2. All the MRSA isolates were resistant to tetracycline and streptomycin.

Table 2

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>9280/t011</th>
<th>21175/t1451</th>
<th>31995/t034</th>
<th>31995/t1451</th>
<th>33141/t011</th>
<th>37122/t11</th>
<th>37126/t11</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>9/0/0</td>
<td>1/0/0</td>
<td>1/0/4</td>
<td>0/0/1</td>
<td>0/0/6</td>
<td>0/0/1</td>
<td>0/0/1</td>
<td>11/0/13</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>9/0/0</td>
<td>1/0/0</td>
<td>5/0/0</td>
<td>1/0/0</td>
<td>6/0/0</td>
<td>1/0/0</td>
<td>1/0/0</td>
<td>24/0/0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>9/0/0</td>
<td>1/0/0</td>
<td>4/1/0</td>
<td>1/0/0</td>
<td>5/1/0</td>
<td>1/0/0</td>
<td>1/0/0</td>
<td>22/2/0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0/0/9</td>
<td>0/0/1</td>
<td>0/0/5</td>
<td>0/0/1</td>
<td>0/0/6</td>
<td>0/0/1</td>
<td>0/0/1</td>
<td>0/0/24</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0/0/9</td>
<td>0/0/1</td>
<td>0/0/5</td>
<td>0/0/1</td>
<td>0/0/6</td>
<td>0/0/1</td>
<td>0/0/1</td>
<td>0/0/24</td>
</tr>
<tr>
<td>Neomycin</td>
<td>8/1/0</td>
<td>1/0/0</td>
<td>4/1/0</td>
<td>1/0/0</td>
<td>6/0/0</td>
<td>0/0/1</td>
<td>0/0/1</td>
<td>20/2/2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>9/0/0</td>
<td>1/0/0</td>
<td>4/1/0</td>
<td>1/0/0</td>
<td>6/0/0</td>
<td>0/0/1</td>
<td>0/0/1</td>
<td>21/1/2</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>8/0/1</td>
<td>1/0/0</td>
<td>5/0/0</td>
<td>1/0/0</td>
<td>6/0/0</td>
<td>1/0/0</td>
<td>1/0/0</td>
<td>23/0/1</td>
</tr>
<tr>
<td>Trimetoprim + sulphamethoxazole</td>
<td>8/0/1</td>
<td>1/0/0</td>
<td>3/0/2</td>
<td>1/0/0</td>
<td>6/0/0</td>
<td>1/0/0</td>
<td>1/0/0</td>
<td>21/0/3</td>
</tr>
</tbody>
</table>

*Results are expressed as the number of susceptible / intermediate / resistant strains

Isolates from two farms were susceptible to erythromycin, while other isolates were resistant. Vancomycin was the only antimicrobial to which all isolates were susceptible. There were 23 strains (96%) susceptible to florfenicol.
The MIC of oxacillin for the isolates was determined, and ranged between 3 and 12 g/L. The oxacillin MIC was relatively low (MIC$_{50}$ 8 mg/L, MIC$_{90}$ 12 mg/L, MIC range 3–16 mg/L).

**Spa typing**

Most of the MRSA isolates belonged to *spa* type t011 (n = 17), which was detected in four out of six farms while *spa* type t034 (n = 5) was detected in one farm. Furthermore, *spa* type t1451 (n = 2) was detected in two farms.

**Discussion**

This is the first study on the presence of MRSA in large pig breeding farms in Croatia. In the framework of the study, dust swabs were taken from facilities of eight large pig farms in Croatia. Positive swabs were found at six out of the eight farms studied.

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 purchasing high-quality breeding animals). This indicates that the MRSA result obtained on the farms cannot be correlated with farm technology.

The MRSA isolates belong to three *spa* types (t011, t034 and t1451), all of which have been detected for the first time in Croatia. T011 and t034 represent the most common *spa* types among European livestock-associated MRSA (LA-MRSA) (Huber et al., 2010), and are highly prevalent *spa* types in Europe with frequencies of 3.6% (t011) and 1.35% (t034). In Switzerland, *spa* type t011 was associated with bovine strains while *spa* type t034 with strains isolated from pigs. In our study, most of the isolates belonged to t011, while the *spa* typing of bovine isolates has never been carried out in Croatia. *Spa* types detected in this study are similar to those detected among swine isolates in Germany (t011, t034, t108, t1451 and t2510) (Köck et al., 2009).

In addition to its resistance against all beta-lactam antibiotics, the typical resistance of LA MRSA also included some other antibiotics used to treat or prevent some bacterial diseases, such as aminoglycosides and macrolides (Vanderhaeghen et al., 2010). Total resistance to tetracycline and streptomycin could be expected from the long-term use of such products at large pig farms in Croatia. A similar result of resistance to tetracycline was obtained by de Neeling et al. (2007) in cases where isolates originated from farms with a long history of tetracycline use. Only two strains were resistant to neomycin and gentamicin, from farms 37122 and 37126. This could indicate the possibility that these strains have the aac(6')-aph(2'') or some other aminoglycoside-modifying enzyme (Vanhoof et al., 1994; Ida et al., 2001), which should be confirmed by further studies.
Vancomycin was the only antimicrobial to which all isolates were susceptible, while 22 (92%) of the strains were susceptible and 2 (8%) were intermediate susceptible to ciprofloxacin, which is in compliance with the results of earlier studies (de Neeling et al., 2007).

In recent years, there has been increasing evidence that pigs can be a source of MRSA for humans (Voss et al., 2005), 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 (Wertheim et al., 2004; de Neeling et al., 2007; Köck et al., 2009). Persons at risk include pig farmers, transporters, slaughterhouse personnel and veterinarians (de Neeling et al., 2007; Köck et al., 2009). A higher prevalence of *S. aureus* carriernship among pig farmers was noted in France (Armand-Lefevre et al., 2005). The results presented here show that MRSA is present on a large number of pig farms in Croatia and that the research should be continued with further tests on animals and people in contact with pigs, to obtain a better insight into the incidence of MRSA infection in pigs and humans.

References


