Occurrence and characterisation of MRSA and extended-spectrum β-lactamases producing *Escherichia coli* isolated from mastitic cows’ milk

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Received: March 9, 2015  Accepted: June 17, 2015

Abstract

The aim of study was the preliminary evaluation of the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) and extended spectrum β-lactamases (ESBL) – producing *Escherichia coli* in 650 milk and inflammatory secretions from cows with clinical or subclinical mastitis. One millilitre of the sample was added to Mueller-Hinton broth supplemented with 6.5% NaCl, Tryptone Soya Broth with cefoxitin and aztreonam, and then to MRSA ID agar. Presumptive MRSA colonies were analysed for the presence of mecA gene. Parallel to MRSA identification, the samples were incubated in buffered peptone water, lauryl tryptose broth and McConkey agar supplemented with cefotaxim for ESBL-producing *E. coli* isolation. These bacteria were identified using API Rapid 32 E and the ability of ESBL production was initially established using disc test D68C and confirmed by MIC technique using Sensititre ESBL plates. The primers (blaCTX, blaTEM, blaSHV, and blaCMY-2-group) for the detection of some of the genes encoding ESBL production were used. The 45 strains of *S. aureus* with mecA gene and 41 strains of *E. coli* with blaTEM gene were detected.

Keywords: cows, mastitis, *Staphylococcus aureus*, *Escherichia coli*, antibiotic resistance.

Introduction

Udder inflammations are still the most frequent and cost affecting diseases of dairy cows in the world (1, 10, 13, 16, 22). *Staphylococcus aureus*, coagulase negative staphylococci, and *Escherichia coli* are the main aetiological agents of clinical and subclinical forms of bovine mastitis (2, 7, 17, 23, 27, 31). Some strains of these bacteria have developed resistance to β-lactam antibiotics, e.g. penicillins, which are used in the treatment of many infections in humans and animals. These strains are methicillin-resistant *S. aureus* (MRSA) and extended-spectrum-β-lactamases (ESBL)-producing *E. coli*. Humans mainly acquire MRSA or ESBL-producing Gram-negative bacteria through direct contact between infected humans, or by contact with medical devices or equipment. Some authors pointed to possible transmission of these pathogens via food-chain (15, 19, 21). MRSA and ESBL-producing *Enterobacteriaceae* family members are especially problematic in hospitals, where a greater risk of infection exists for patients with weakened immune systems than for the general public. MRSA has been reported to occur in food-producing animals, most often in intensively reared pigs, veal calves and chickens, and has also been found in horses and companion animals (14, 15, 25). The emergence of MRSA in dairy cattle may be associated with contact with other host species, or with the exchange of genetic material between *S. aureus* and coagulase negative *Staphylococcus* sp., which are the most common species associated with bovine intramammary infections and commonly carry antimicrobial resistance determinants. The occurrence of ESBL-producing bacteria in cattle has also been noted (9, 18, 23), but there is no sufficient data about their occurrence in mastitic cows’ milk. The paper presents the results of the study on the occurrence and
characterisation of MRSA and ESBL-producing *E. coli* in milk and inflammatory secretions from mastitic bovine mammary gland.

**Material and Methods**

**Inflammatory secretion/milk samples.** Inflammatory secretion of quarter milk samples from cows with subclinical or clinical forms of mastitis were collected (*n* = 650). The first few streams of milk were discarded and then 5-10 mL of the secretion were collected into sterile 10 mL tubes. The samples were cooled and transported to laboratory within 24 h or were kept frozen for a maximum of one month, prior to laboratory investigation.

**MRSA isolation.** One millilitre of the sample was added to 49 mL of Mueller-Hinton broth supplemented with 6.5% NaCl and incubated at 37°C for 16-20 h. One millilitre of the enriched Mueller-Hinton broth was inoculated into 9 mL of Tryptone Soya Broth + 3.5 mg/L of cefoxitin and 75 mg/L of aztreonam, and incubated for further 16-20 h at 37°C. One loop-full (10 µL) of TSB was spread onto an MRSA ID agar (bioMérieux, France) and a blood agar plate, and incubated for 24-48 h at 37°C. Based on colony morphology and colour, presumptive pink MRSA colonies on the MRSA ID agar were subcultivated on blood agar and further analysed for peroxidase activity by dripping a drop of 3% H₂O₂. Presumptive MRSA colonies, which produce tiny bubbles, were used for DNA extraction.

**MRSA DNA extraction.** One individual colony of each strain was suspended in 50 µL of lysis buffer amended with lysostaphin (30 µL/L) and lysozyme, and heated at 56°C for 30 min. Next the suspensions were heated at 99°C for 5 min, chilled on ice, and then centrifuged at 13 000 × g for 1 min to pellet the cellular debris. The supernatant (5 µL) was subsequently used as a source of DNA template. The purity and concentration of DNA preparations were measured spectrophotometrically at 260 and 280 nm (GeneQuant 1300, GE Healthcare).

**MRSA PCR assays.** Each DNA amplification was performed in the 24 µL reaction mixture consisting of: DNA template; 1X PCR buffer (Fermentas); 200 µM of dNTPs; 4 mM MgCl₂; 1 U of Taq DNA polymerase; nucleotide primers, and water. The sequences and characteristics of the primers used in this study are shown in Table 1.

Multiplex PCRs (for *mecA*, *nuc* and 16S *rRNA* genes) were performed in a thermocycler (T3, Biometra) under the following conditions: initial DNA denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1. The final extension step was done at 72°C for 10 min. After staining with ethidium bromide for 0.5 min and washing in distilled water, the gels were photographed under UV light. The sizes of the PCR amplicons were compared to the 100 bp DNA marker (Fermentas).

**ESBL-producing *E. coli* isolation.** Ten millilitres of sample were incubated overnight at 37 ± 1°C with 90 mL of buffered peptone water. One millilitre of culture was transferred to 9 mL of lauryl tryptose broth (Oxoid, UK) and incubated for 48 h at 37 ± 1°C. One loop (10 µL) of the culture was spread on the surface of McConkey agar (Oxoid, UK) supplemented by cefotaxim (2 mg/L), and incubated at 18-24 h at 37 ± 1°C. The bacteria forming colonies typical for *E. coli* were identified using API Rapid 32 E test (bioMérieux, France), according to manufacturer’s instruction. The ability of ESBL production was initially established using disc test D68C (Mast Diagnostica, Germany), and confirmed by MIC technique, using Susitite ESBL plates (Trek Diagnostic Systems, USA).

**ESBL-producing *E. coli* DNA extraction.** DNA of *E. coli* was obtained using heat-shock method. Two colonies of the selected strain were suspended in 200 µL of phosphate buffered saline (PBS, Thermo Scientific, USA) and boiled for 10 min. After cooling, the mixture was centrifuged at 10 000 rpm for 20 s. The supernatant was diluted in deionised water (1:10) and used for PCR.

**ESBL-producing *E. coli* PCR assays.** The primers used for the detection of some of the genes encoding ESBL production (*blaCTX*, *blaTEM*, *blashv*, and *blacmv2-group*) were described before (29), and are presented in Table 2. The amplification was carried out in a 25 µL volume containing 12.5 µL of Maxima Master Mix (Thermo Scientific), 0.1 µL of each primer, 11.3 µL of water, and 1 µL of DNA solution. The PCR was initiated by 5 min of denaturation at 95°C followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at the temperature appropriate for each primer for 30 s, and extension for 30 s at 72°C. The last extension step was prolonged to 10 min. The products of reaction were analysed by electrophoresis in 1.5% agarose gel containing ethidium bromide.

### Table 1. Sequences and characteristics of the primers used in the MRSA identification

<table>
<thead>
<tr>
<th>Primer sequence (5′→3′)</th>
<th>Target gene</th>
<th>Annealing temp. (°C)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGG ATC ATA GCC TCA TTA TTC</td>
<td><em>mecA</em></td>
<td>52</td>
<td>527</td>
</tr>
<tr>
<td>AAC GAT TGT GAC AGC ATA GCC</td>
<td></td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>TCA GCA AAT GCA TCA CAA ACA G</td>
<td><em>nuc</em></td>
<td>55</td>
<td>255</td>
</tr>
<tr>
<td>CGT AAA TGC ACT TGC CTT CAG G</td>
<td></td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>GTG CCA GCA GCC GCG GTA A</td>
<td>16S rRNA</td>
<td>66</td>
<td>886</td>
</tr>
<tr>
<td>AGA CCC GGG GAA CGT ATT CAC</td>
<td></td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>

![Image of the page](image-url)
The presence of the nuc gene, typical for nuclease gene in *S. aureus* and not present in e.g. *S. hyicus* nor other coagulase-positive genes, generating the amplicon of 255 bp, was observed in all DNA samples. These samples were chosen after the primary identification of morphology and presumptive MRSA colour on MRSA ID agar. All strains examined were mecA positive, generating the amplicon of 527 bp, characteristic for methicillin-resistant *S. aureus*. As the internal control, the PCR amplification of examined samples showed the presence of 16S rRNA gene, generating the amplicon of 886 bp. For all 41 strains identified as *E. coli* and on the basis of growth on MacConkey-CTX medium, disc test D68C and MIC results evaluated as ESBL positive, the presence of the *blaTEM* gene was observed. The genes *blaCTX, blaSHV*, and *blaCMY-2* were not detected.

### Discussion

Staphylococci are common aetiological agents of bovine mastitis and infections caused by methicillin-resistant staphylococci are not only more difficult to treat, but may also pose a risk to public health. MRSA was first reported in cows in 1972 in Belgium and has been sporadically reported until now. Methicillin resistance is caused by mecA gene, which encodes the penicillin-binding protein 2a with decreased affinity for β-lactam antibiotics. Methicillin-resistant *S. aureus* should be considered as resistant to all penicillins, cephalosporins, cepham, and other β-lactams such as ampicillin-sulbactam, amoxicillin-clavulanic acid, piperacillin-tazobactam, and carbapenems, regardless of the in vitro test results obtained with these agents (27).

In the last five decades, methicillin-resistant *S. aureus* has spread as human hospital-acquired pathogens (HA-MRSA) throughout the world. More recently, community-acquired (CA-MRSA) and livestock-associated (LA-MRSA) have emerged. The MRSA isolations from livestock such as cattle, pigs or poultry have been increasingly reported and possible transmission to humans has already been demonstrated (3-5, 9, 25).

In our study, we focussed on MRSA identification. We found that among 650 samples tested, 45 were mecA positive. This is the first study on the presence of MRSA in bovine mastitis cases in Poland. In the previous study by Malinowski et al. (18), coagulase-negative *Staphylococcus* sp. strains were isolated from normal (9.4%) and inflamed quarter milk samples of dairy cows in one herd localised in southern Poland. In the study of Gindonis et al. (12), MRSA was a rare finding in bovine mastitis in Finland. Only two out of 135 (1.5%) *S. aureus* isolates were positive for mecA genes. On the other hand, in coagulase-negative staphylococci (CoNS) originated from bovine mastitis, methicillin resistance was more common. In the two CoNS collections studied, 5.2% (17/324) and 1.8% (2/110) of the isolates were mecA positive. In earlier studies, the occurrence of MRSA (percentage of mecA-positive isolates out of all isolates in the given *S. aureus* collection) from milk from Japan, Switzerland, Korea, and Belgium has been 1.1%, 1.4%, 1.6% and 9.3% respectively (12, 28). In the study of Bardiau et al. (3), among 430 *S. aureus* isolates collected from bovine mastitis cases, 19 (4.4%) isolates were identified as MRSA, and the presence of the mecA gene was confirmed by PCR for all 19 isolates. The aim of the study of Turkyilmaz et al. (27) was to identify MRSA in milk samples gathered from 2002 to 2006 in Aydin region in Turkey. Among 93 *S. aureus* strains isolated from bovine milk with mastitis, 16 were resistant to methicillin.

Similar as MRSA, Gram-negative bacilli, able to produce extended-spectrum β-lactamases (ESBL or AmpC), are an important problem in medicine. The occurrence of these bacteria in food animals and in farm environment was frequently noted (6, 11, 20, 29, 30). Although *E. coli* is one of the most important causative factors of mastitis, there is only little information about their ability for ESBL production. According to Dahmen et al. (8) among 1342 isolates of *E. coli* from mastitis cases in France, only 5 (0.3%) were ESBL positive. The occurrence of ESBL-producing *E. coli* in the milk of cows with acute mastitis was also described by Su et al. (24).

### Table 2. Sequences and characteristics of the primers used in the ESBL identification

<table>
<thead>
<tr>
<th>Sequence (5’→3’)</th>
<th>Target gene</th>
<th>Annealing temp. (°C)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGTGCAGYACCATGATAGTGC</td>
<td><em>blaCTX</em></td>
<td>60</td>
<td>593</td>
</tr>
<tr>
<td>TGGTACTARGTSCCGAAGYCAAGG</td>
<td><em>blaCTX</em></td>
<td>53</td>
<td>861</td>
</tr>
<tr>
<td>TGAGTATTCAACATTTCCGTGT</td>
<td><em>blaTEM</em></td>
<td>53</td>
<td>937</td>
</tr>
<tr>
<td>TTACCAATGCCTTAATCAGTGA</td>
<td><em>blaTEM</em></td>
<td>60</td>
<td>758</td>
</tr>
<tr>
<td>CAAAACCGCGGTTATTC</td>
<td><em>blaSHV</em></td>
<td>53</td>
<td>937</td>
</tr>
<tr>
<td>TTAGGCTGCGACGTCT</td>
<td><em>blaSHV</em></td>
<td>60</td>
<td>758</td>
</tr>
<tr>
<td>GCACCTAGCCTATACGGCAG</td>
<td><em>blaCMY-2</em></td>
<td>60</td>
<td>758</td>
</tr>
<tr>
<td>GCTTTTCAAGAATGCGCCAG</td>
<td><em>blaCMY-2</em></td>
<td>60</td>
<td>758</td>
</tr>
</tbody>
</table>

**Results**

The presence of the nuc gene, typical for nuclease gene in *S. aureus* and not present in e.g. *S. hyicus* nor other coagulase-positive genes, generating the amplicon of 255 bp, was observed in all DNA samples. These samples were chosen after the primary identification of morphology and presumptive MRSA colour on MRSA ID agar. All strains examined were mecA positive, generating the amplicon of 527 bp, characteristic for methicillin-resistant *S. aureus*. As the internal control, the PCR amplification of examined samples showed the presence of 16S rRNA gene, generating the amplicon of 886 bp. For all 41 strains identified as *E. coli* and on the basis of growth on MacConkey-CTX medium, disc test D68C and MIC results evaluated as ESBL-positive, the presence of the *blaTEM* gene was observed. The genes *blaCTX, blaSHV*, and *blaCMY-2* were not detected.
Timofte et al. (26) isolated *E. coli* with *blaCTX-M-15* and *blaTEM-1* genes from the cases of bovine mastitis in United Kingdom. Ohnishi et al. (20) described 65 ESBL-producing *Enterobacteriaceae* (*E. coli* and *Klebsiella pneumoniae*) with *blaCTX-M* isolated from 258 880 samples of mastitic milk samples. There are no published data from Poland. The results of this study confirmed the occurrence of MRSA and ESBL-producing *E. coli* in mastitic cow milk samples and suggest a potential risk of transmission from cows with mastitis to humans such as veterinarians or farmers.

Conflict of Interests Statement: The authors declare that there is no conflict of interest regarding the publication of this article.

Financial Disclosure Statement: This scientific work was financed from science funds in 2009 - 2012 as a research project No. NN308096737.

Animal Rights Statement: The authors declare that the experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

References


