Characterisation of *Staphylococcus aureus* and *Staphylococcus aureus*–like strains isolated from table eggs

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Abstract

The aim of the study was to provide a detailed phenotypic and genotypic characterisation of *Staphylococcus aureus* strains and the group of microorganisms with unusual biochemical patterns (called *Staphylococcus aureus*-like) isolated from table chicken eggs. All of the strains tested exhibited resistance to at least one of the 17 antibiotics tested, and 55.55% of isolates were found to be resistant to five or more of them. PCR used for detection of the methicillin resistance gene (mecA) confirmed the presence of a specific product of 533 bp in the case of two of the isolated *S. aureus*-like strains. Analysis of the phylogenetic relationship between eight of *S. aureus* and ten *S. aureus*-like strains distinguished 18 macrorestriction profiles following digestion with *SmaI* endonuclease, indicating that there were no identical strains with the same macrorestriction profile. However, the presence of methicillin-resistant strains indicates a serious risk to consumer health.

Key words: table eggs, *Staphylococcus aureus*, antibiotic resistance, tube coagulase test, PFGE.

Introduction

The presence of *Staphylococcus* bacteria in food, particularly in raw meat products or poultry products, is one of the most common causes of food poisoning worldwide. In Poland, as many as 793 cases of food poisoning caused by *Staphylococcus* bacteria were noted between 2004 and 2010, nearly half of them occurred in 2009 and 2010 (3). The type of food, which is most frequently the source of food poisoning, varies according to geographical location, due to differences in eating habits of populations of particular regions. Data indicates that the most common way of transmission of enterotoxigenic strains is food made of meat, poultry, and mixed products of animal origin. Reports demonstrate that *Staphylococcus aureus* is responsible for the majority of staphylococcal infections in humans in many countries. This is due to certain characteristics of this species, the most important of which is the ability to produce coagulase, thermostable nuclease, or enterotoxins (5, 9). Standard microbiological identification of *S. aureus* is based on its ability to clump in plasma via the activity of clumping factor (bound coagulase) (CF) and coagulase (free coagulase) (2). The simultaneous production of CF and coagulase is a characteristic feature of most *S. aureus* isolates, and plays an important role in species classification and microbiological diagnosis (17). Coagulase is encoded by the gene *coa*, which is localised between the genes of lipase (*geh*) and protein A (*spa*) in the chromosome of *S. aureus*. Expression of the coagulase gene, like that of protein A, is coded by the regulatory region *agr* (10).

Within the *S. aureus* species substantial diversity is observed among the microorganisms, which are isolated from different sources at different times and from different geographical regions, and can be classified into many different types or strains (13). Of the greatest concern are infections induced by *S. aureus* strains belonging to the human ecovar and antibiotic-resistant, particularly methicillin-resistant (MRSA) strains (25). It has been established that MRSA strains of *S. aureus* can be transmitted occasionally from humans to animals or to food products of animal origin, which supports the need for research aimed at a detailed characterisation of these strains. There is also...
increasing awareness of existence of the *S. aureus* strains, which are negative in coagulase tube-test or clumping factor-negative. Such strains are commonly referred to as atypical *S. aureus*, and their isolation from clinical and animal specimens occurs with increasing frequency. Some of these atypical strains contain the enterotoxin genes (18, 24).

The aim of the study was to provide a detailed phenotypic and some genotypic characterisation of *S. aureus* strains, and the group of microorganisms with an unusual biochemical patterns (referred here as *S. aureus*-like) isolated from table chicken eggs.

### Material and Methods

**Material.** Ninety table chicken eggs from nine different battery cages laying farms situated in the vicinity of Lublin, purchased in shops in the city of Lublin were used in this study.

**Bacteriological testing.** The samples (whites, yolks, and shells) were pre-enriched in buffered peptone water (Buffered Peptone Water, Biocorp, Poland) at 37°C for 18-24 h. The samples were then transferred onto blood agar (Blood LAB-AGAR, Biocorp, Poland) and the selective medium MSA (Mannitol Salt LAB-AGAR, Biocorp, Poland), and then incubated under aerobic conditions at 37°C for 24-48 h, depending on the rate of growth of the bacteria. Single colonies were transferred onto blood agar to isolate pure bacterial cultures, and an initial bacteriological characterisation was performed by evaluating the morphology of the colonies and the presence and type of haemolysis. *Staphylococcus* strains isolated from the eggs were maintained frozen in 15% glycerol at -80°C.

**Identification of *Staphylococcus* strains.** API Staph (BioMerieux, France), which includes 19 biochemical tests, was used to identify the isolated *Staphylococcus* strains. Additional tests were performed as well: a free coagulase test (BioMerieux, France); tests for bound coagulase (clumping factor) and surface protein A (SlideX Staph-Kit (BioMerieux, France); a DNase test conducted on commercially-prepared DNA agar (BIOCORP, Poland); a catalase test, using bacterial cultures transferred onto tryptic soy agar (Tryptic Soy Agar–TSA, BioMerieux, France); and a β-galactosidase activity assay, using the commercial kit API ZYM (BioMerieux, France) according to the producer’s recommendations.

**Determination of the susceptibility of the bacteria to selected chemotherapeutics.** Susceptibility of the isolated bacterial strains to selected antibiotics and sulphonamides was tested using the Kirby-Bauer disk diffusion method on Mueller-Hinton medium (bioMerieux), in accordance with international norms (6). The results were read and interpreted based on the diameter of the zone of inhibition. The strains were designated as resistant (R) or susceptible (S). The susceptibility profiles of the bacteria were determined for the following chemotherapeutics (OXOID, U.K.): amoxicillin, amoxicillin with clavulanic acid, cephalixin, chloramphenicol, enrofloxacin, erythromycin, flumequine, gentamicin, clindamycin, lincomycin/spectinomycin (Linco-Spectin), neomycin, oxacinillin, oxytetracycline, penicillin G, streptomycin, trimethoprim/sulfamethoxazole, and tetracycline.

**Detection of meca by PCR.** All isolates were tested for the presence of meca gene using PCR. Reference strains of *S. aureus*—methicillin-susceptible (ATCC 29213) and methicillin-resistant (ATCC 43300) – were used as controls. Primers complementary to the conserved region in the meca gene, enabling amplification of 533 bp fragment in length were used for the amplification (4). The PCR products were analysed by electrophoresis in 1.5% agarose gel (Sigma Aldrich, USA) prepared in TBE buffer, in the presence of a molecular weight standard (100 bp DNA, Fermentas, Lithuania).

**Pulsed-field gel electrophoresis (PFGE).** Gene polymorphism of the eight strains isolated, within the species *S. aureus* (SA) and ten within *S. aureus*-like (SAL) strains were analysed using pulsed field gel electrophoresis according to Shimizu *et al.* (22). DNA fragments digested with *Sma*I endonuclease were separated by electrophoresis in 1% agarose (Sigma Aldrich, USA) in a pulsed electric field (PFGE). The separation was carried out in 0.5 × TBE buffer at 14°C; initial pulse time ~5 s; final pulse time ~40 s; voltage 6 V/cm; time 23 h in a CHEF-DR II system (BioRad). To compare the size of the digested genome fragments, a DNA molecular weight marker was used (Pulse Marker™, 50-1000 kb, Sigma Aldrich, USA). DNA fragments were visualised after staining with ethidium bromide (5 µg/mL) for 20 min using a UV transilluminator. The image was registered and analysed using Quantity One® software ver. 4.1 (BioRad). The results were analysed statistically with NTSYS-pc cluster analysis software (NTSYS-pc ver. 2.02g, Applied Biostatistics Inc. 1986-1998). Genetic similarity of the strains (SI—similarity index) was calculated using Dice’s coefficient ($S_D$). Cluster analysis was performed by UPGMA, and dendograms were constructed according to Priest and Austin (20).

### Results

**Isolation and identification of bacterial strains.** A total of 105 bacterial strains identified as *Staphylococcus* were isolated from the samples, of which eight (7.61%) isolates belonged to the species *S. aureus* (SA1 to SA8) and 10 isolates, giving negative result in tube coagulase test were named *S. aureus*-like (SAL1 to SAL10). Five strains of *S. aureus* were isolated from the shells and three from the yolks. The
largest number (five) of *S. aureus*-like strains were also isolated from shells but three of them were isolated from whites and two from yolks.

The *S. aureus* strains exhibited biochemical reactions typical for the species. All isolates (eight) produced protein A and clumping factor and synthesised detectable quantities of coagulase in the tube coagulase test. Four strains exhibited \( \beta \)-haemolysis, while the other four exhibited \( \alpha \)-haemolysis. Strong activity of DNase and \( \beta \)-galactosidase was observed in six and two *S. aureus* isolates, respectively. None of the strains was found to acidify methyl-\( \alpha \)-D-glucopyranoside, melibiose, or xylitol.

All *S. aureus*-like strains were Gram-positive cocci in clusters, catalase positive, API20 Staph and slide coagulase test positive, and produced protein A, but tube coagulase test were negative. Three strains exhibited \( \beta \)-haemolysis, while the other seven exhibited \( \alpha \)-haemolysis. Strong activity of DNase and \( \beta \)-galactosidase was observed in three isolates. None of the strains was found to acidify methyl-\( \alpha \)-D-glucopyranoside, melibiose, or xylitol. Detailed data is presented in Table 1.

**Determination of the susceptibility of the isolated bacteria to selected antibiotics.** All of the eight *S. aureus* and ten *S. aureus*-like strains exhibited resistance to at least one of the 17 antibiotics tested. Eight of the *S. aureus* and six of *S. aureus*-like strains were resistant to five or more antibiotics. The largest number of strains were resistant to erythromycin (12), tetracycline (12), oxytetracycline (11), penicillin G (9), and amoxicillin (8). None of the 18 (*S. aureus* and *S. aureus*-like) strains exhibited resistance to cloramphenicol, gentamicin, cephalaxin, or amoxicillin with clavulanic acid. Detailed data is presented in Table 2.

**Detection of mecA by PCR.** The results of PCR to detect the methicillin resistance gene (mecA) confirmed the presence of a specific amplicon of a 533 bp band in two of *S. aureus*-like strains, designated as SAL1 and SAL2 (Fig. 1).

**Pulsed-field gel electrophoresis (PFGE).** In the macrorestriction profiles obtained, following enzyme digestion, 10 to 22 DNA fragments were present, which ranged from 49kb to 615kb (Figs 2, 3). Analysis of the phylogenetic relationship between eight of *S. aureus* and ten *S. aureus*-like strains distinguished 18 macrorestriction profiles following digestion with *Smal* endonuclease. No identical strains with the same macrorestriction profile were observed. The genotypic similarity of all examined strains was analysed statistically with UPGMA NTSYS software and expressed as Dice’s coefficient, and in the form of a dendrogram (Fig. 4). The phylogenetic similarity of the profiles ranged from about 3% to 86%.
Table 1. Biochemical properties of the isolated *Staphylococcus aureus* and *Staphylococcus aureus*-like strains

<table>
<thead>
<tr>
<th>Property</th>
<th><em>S. aureus</em> n = 8</th>
<th><em>S. aureus</em>-like = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>pigment production</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>coagulase</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>β hemolysis</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>α hemolysis</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>DNase</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>catalase</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>clumping factor</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>protein A</td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

Acid production from:

- glucose: 8 - 10
- fructose: 8 - 10
- mannose: 5 - 6
- maltose: 7 - 9
- lactose: 6 - 8
- trehalose: 7 - 10
- mannotol: 6 - 8
- raffinose: 0 - 1
- saccharose: 8 - 10
- NAG*: 6 - 10
- MDG**: 0 - 0
- melibiose: 0 - 0
- xylitol: 0 - 0
- xylose: 0 - 1

Production of:

- urease: 5 - 7
- ADH***: 6 - 10
- β-galactosidase: 2 - 3
- alkaline phosphatase: 8 - 10
- nitrate reduction: 8 - 10
- acetoin****: 8 - 7

* N-acetylglucosamine, ** methyl-α-D-glucopyranoside, *** production of arginine dihydrolase, **** Voges-Proskauer test

Table 2. Patterns of antibiotic* resistance in the isolated *Staphylococcus aureus* and *Staphylococcus aureus*-like strains, taking into account methicillin-resistant strains

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Antibiotic resistance pattern*</th>
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<tbody>
<tr>
<td>ATCC 43300, MRSA</td>
<td>AML, AMC, CL, ENR, E, CN, DA, LS, N, OX, P, S, SXT</td>
</tr>
<tr>
<td>ATCC 29213, MSSA</td>
<td>AML, C, OX, P, S, TE</td>
</tr>
<tr>
<td>SA1</td>
<td>E</td>
</tr>
<tr>
<td>SA2</td>
<td>E</td>
</tr>
<tr>
<td>SA3</td>
<td>AML, E, N, OT, P, TE</td>
</tr>
<tr>
<td>SA4</td>
<td>AML, E, DA, OT, P, TE</td>
</tr>
<tr>
<td>SA5</td>
<td>AML, UB, DA, LS, OT, P, TE</td>
</tr>
<tr>
<td>SA6</td>
<td>P, SXT</td>
</tr>
<tr>
<td>SA7</td>
<td>OT, OX, TE</td>
</tr>
<tr>
<td>SA8</td>
<td>AML, E, UB, DA, N, OX, P</td>
</tr>
<tr>
<td>SAL1**</td>
<td>AML, E, ENR, P, S, TE</td>
</tr>
<tr>
<td>SAL2**</td>
<td>AML, E, ENR, OT, P, S, TE</td>
</tr>
<tr>
<td>SAL3</td>
<td>E, OT, TE</td>
</tr>
<tr>
<td>SAL4</td>
<td>AML, E, ENR, UB, OT, P, S, TE</td>
</tr>
<tr>
<td>SAL5</td>
<td>E, ENR, DA, OT, TE</td>
</tr>
<tr>
<td>SAL6</td>
<td>ENR, UB, LS, OT, TE</td>
</tr>
<tr>
<td>SAL7</td>
<td>OT, TE</td>
</tr>
<tr>
<td>SAL8</td>
<td>AML, OT, OX, P, TE</td>
</tr>
<tr>
<td>SAL9</td>
<td>E</td>
</tr>
<tr>
<td>SAL10</td>
<td>E</td>
</tr>
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</table>


** strains which showed the presence of the meca gene responsible for resistance to methicillin
Discussion

The requirements for the table eggs include high nutritional value and digestibility as well as safety of use in the everyday diet of millions of people worldwide. Taking these criteria into account, the presence of pathogenic bacteria in food, including table chicken eggs, may pose a serious health problem (food poisoning and foodborne infections), considering the fact that many foods containing eggs or egg products undergo no thermal treatment, or thermal treatment insufficient to neutralise these pathogens (3, 21). The present study revealed a moderately high rate of Staphylococcus contamination of table chicken eggs, with S. aureus accounting for 7.6% of isolates and 9.5% strains, which showed extremely close correlation to S. aureus with the exception of coagulase production. One of the typical features that distinguish the more pathogenic Staphylococcus strains from the less pathogenic is the ability to produce free coagulase and bound coagulase (clumping factor). This feature is also considered as one of the virulence factors (2, 17).

Detailed analysis of the biochemical and enzymatic properties of the isolated S. aureus strains demonstrated that only eight out of the 18 isolated strains produced coagulase in the tube coagulase test. Moreover, both of the methicillin-resistant strains were coagulase-negative. Although the available literature shows that food poisoning in humans is usually caused by coagulase-positive strains of S. aureus, many authors have also emphasised that enterotoxicity can occur among coagulase negative strains (CNS) (7, 8, 14). Although ten strains isolated from table chicken eggs were negative in tube coagulase test, all of them were positive for CF, protein A, and thermonuclease. The result of biochemical tests obtained for tested strains allowed classifying them as S. aureus. However, they are called S. aureus-like strains because of the need to reaffirm their belonging to the species S. aureus. For this purpose, further molecular identification of the isolates, which included sequencing of 16S rRNA gene and RT-PCR of coagulase (coa) is needed (1). In recent years, several authors reported a significant role of atypical (free-coagulase negative) S. aureus infections in animals and humans. The incidence of coagulase-or CF-deficient strains is estimated to be between 1% and 20%, or between 5% and 15%, respectively, of all S. aureus isolates (16). Available data indicates that these organisms are often aetiological agents in subclinical form of udder inflammations in cattle (18). It is worth noting that in the present study, the ability of microorganisms to produce a free coagulase was examined in strains that had been stored in a frozen glycerol. Wilkinson et al. (26) observed that such a method of storage may affect the result of examination of the strains for the presence of free coagulase. They found that when the inocula of strains were taken from brain heart infusion agar, positive
coagulase reactions were noted. However, when the inocula were taken from frozen glycerol cultures, none of these strains gave positive coagulase test. Lotter and Genigeorgis (15) have reported the isolation of coagulase-positive variants from cultures of coagulase-negative, enterotoxigenic staphylococci transferred on brain heart infusion agar at least nine times.

While all the S. aureus and S. aureus-like strains tested in the present study were susceptible to amoxicillin with clavulanic acid, cephalaxin, chloramphenicol, and gentamicin, half of S. aureus (four) and six S. aureus-like strains were found to be resistant to five or more antibiotics. Among the strains of S. aureus, the most frequently observed resistance patterns included lack of susceptibility to amoxicillin, penicillin G, erythromycin, tetracycline, and oxytetracycline. All strains S. aureus-like, which were resistant to at least five antibiotics showed resistance to tetracycline and most of them were resistant to amoxicillin, penicillin G, erythromycin, enrofloxacin, and oxytetracycline. The present study confirmed the presence of the mecA gene responsible for resistance to methicillin in two out of the 10 S. aureus-like strains, which were present in the yolk and white of two different chicken eggs from the same source (Fig. 1).

From an epidemiological point of view, this data is especially alarming. The two methicillin-resistant strains were also resistant to six and seven of the antibiotics (Table 2). Resistance to methicillin is heterogeneous, which can cause some difficulty in determining these characteristics under in vitro conditions, and may lead to complications in preparation of the test and interpretation of results (11, 12). Most multiresistant strains of S. aureus, and in particular MRSA strains, exhibit a lack of resistance to aminoglycoside antibiotics, macrolides, chloramphenicol, tetracyclines, and fluoroquinolones. Moreover, MRSA strains are also considered to be resistant to all cephalosporins and other β-lactam antibiotics regardless of the results of tests conducted under in vitro conditions (6). Some authors reported isolation of S. aureus strains being negative in the expression of coagulase gene, especially among MRSA strains. This lack of expression of coagulase gene was explained as polar result of insertion of a genetic element containing the mecA gene into the bacterial chromosome near the coagulase and protein A genes. Lack of plasma clotting may be the result of a complete inactivation of the coa gene. It is also possible that production occurs below the level of sensitivity of the method used for detection of the enzyme (19).

DNA restriction analysis using pulsed field gel electrophoresis (PFGE) revealed eighteen different patterns, which means that in the case of S. aureus and also S. aureus-like there were no identical strains with the same macrorestriction profile (Figs 2, 3). Strains in order to belong to one genotype, must be 100% identical in terms of the number and arrangement of fragments resulting from digestion of the genome and electrophoresis (23). Therefore, in this study, 18 macrorestriction profiles were distinguished among the eight S. aureus and ten S. aureus-like strains isolated from the contents and shells of table chicken eggs (Fig. 4). This indicates that the strains originated from different sources.

Based on the results of the study it is not possible to definitively determine whether the presence of S. aureus in the chicken eggs could have been caused by contamination of the eggs during their preparation for sale or by the presence of the bacteria in the flock of laying hens from which the material originated. Nevertheless, the presence of methicillin-resistant strains indicates a real risk to consumer’s health. The results presented support the need for further research on S. aureus strains isolated from chicken eggs, particularly in regard to their enterotoxicity, biotype, and ecological origin, which may enable determination of the source of the strains and how these infections are spread. In the case of S. aureus-like strains, confirmation of their membership in the species is needed. Moreover, it can be concluded that there should be awareness of the existence of atypical S. aureus strains because in routine bacteriological testing these strains can easily be misclassified as non-aureus staphylococci.

References

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