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Original article

Plasmid profile analysis and evaluation of antibiotic susceptibility of *Staphylococcus aureus* strains isolated from table chicken eggs

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Abstract

The aim of this study was to isolate and characterize *Staphylococcus aureus* bacteria present on the shell surfaces and in the contents of chicken eggs, taking into account their phenotypic properties, antibiotic susceptibility patterns, and the presence of plasmid DNA. The study included 90 table chicken eggs from laying farms situated in the vicinity of Lublin. A total of 105 bacterial strains identified as *Staphylococcus* were isolated from the material, of which 18 (17.14%) were of the species *Staphylococcus aureus.* All 18 *S. aureus* strains were found to be resistant to at least one of the antibiotics tested, while some (55.55%) showed resistance to five or more of the 17 therapeutic agents. The greatest number of strains showed resistance to erythromycin (66.66%), tetracycline (66.66%), oxytetracycline (61.11%), penicillin G (50%), and amoxicillin (44.44%). The plasmid profile analysis of the *S. aureus* strains made it possible to evaluate the dependence between antibiotic susceptibility and the presence of plasmids in particular isolates. The results showed that plasmids in various quantities and of varying molecular weights were isolated from 17 of the strains. Most often isolated were small plasmids, of 5.6 kb – from 11 of the *S. aureus* strains (61.11%), 2.5 kb – from 9 strains (50%), 4.1 kb – from 8 (44.44%), and 4.6 kb – from 7 (38.88%) of the strains.

Key words: table eggs, *Staphylococcus aureus* contamination, antibiotic resistance, plasmids

Introduction

Nearly half of known *Staphylococcus* species are indigenous to the human organism and/or that of other animal species. Most are saprophytes which are part of the bacterial flora present in humans and animals. The disease processes they induce are the result of many complex mechanisms determining pathogenicity. Data from the literature show that clumping factor (CF) is present on the cell surface of as many as 50% of pathogenic strains with the ability to multiply in human serum (Różalska et al. 1995). The species most often isolated from food products of animal origin is *Staphylococcus aureus*, which is isolated with equal frequency from personnel engaged in the processing, storage, and transport of these products. Most strains of this species have the ability to produce one or more enterotoxins, which in many cases are the cause of serious food poisoning in humans who have eaten food contaminated with these

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bacteria (Hatakka et al. 2000, Tamarapu et al. 2001). In terms of treatment of infections caused by staphylococci, what is most important is knowledge of the dynamics of phenotypic and genotypic changes, and in particular, of acquisition of resistance to antibiotics. This trait is associated with the singular adaptive plasticity of staphylococci and their defence mechanisms. This results in part from the ability to acquire new genetic information in the form of extrachromosomal plasmid DNA or via transposable genetic elements such as transposons and integrons, or mutations within chromosomal genes. Most naturally occurring strains of *S. aureus* have one or more plasmids. Data can be found in the available literature on the dependence between resistance of *Staphylococcus aureus* to antibiotics or heavy metal ions and the presence of plasmids which give the host cell these phenotypic traits (Adeleke and Odelola 1997, Łopaciuk and Dzierżanowska 2002).

Plasmid DNA analysis enables bacterial isolates to be differentiated according to the quantity and size of their plasmids. The main problem with plasmid analysis is a lack of stability and reproducibility. The mobility characteristic of extrachromosomal DNA means that plasmids can be easily acquired or lost, in both *in vivo* and *in vitro* conditions (Hartstein et al. 1995).

The threats posed by the properties of staphylococci encountered in the environment and those isolated from animals or food products of animal origin, including poultry and table chicken eggs, substantiate the necessity of research including both their frequency of occurrence and a detailed characterization of *Staphylococcus aureus* bacterial strains, which often pose a threat to human health (Przybylska 2001).

The aim of the study was to isolate and characterize *Staphylococcus aureus* bacteria present on the shells of table chicken eggs and in their contents, taking into account their phenotypic properties, antibiotic susceptibility patterns, and the presence of plasmid DNA.

Materials and Methods

Material for the study

The study included 90 table chicken eggs from 9 laying farms situated in the vicinity of Lublin (Poland), purchased in shops in the city of Lublin.

Two reference strains, methicillin-resistant *Staphylococcus aureus* (ATCC 43300, MRSA) and methicillin-susceptible *Staphylococcus aureus* (ATCC 29213, MSSA), obtained from the Central Laboratory of Sera and Vaccines in Warsaw (Poland), were used as a control.

Bacteriological testing

The material (whites, yolks and shells) was pre-enriched in buffered peptone water (Buffered Peptone Water, Biocorp, Poland) at 37°C for 18-24 hours. The material was then transferred onto blood agar (Blood LAB-AGAR, Biocorp, Poland) and the selective medium MSA (Mannitol Salt LAB-AGAR, Biocorp, Poland), and incubated in aerobic conditions at 37°C for 24-48 hours, depending on the rate of growth of the bacteria. Single colonies were then transferred onto blood agar to isolate pure bacterial cultures, and an initial bacteriological characterization was performed by evaluating the morphology of the colonies and the presence and type of hemolysis.

Identification of Staphylococcus strains

API Staph (BioMerieux, France), which includes 19 biochemical tests, was used to identify the isolated *Staphylococcus* strains. Additional tests were also performed: a free coagulase test (bioMerieux, France); tests for bound coagulase (clumping factor) and surface protein A – Slidex Staph-Kit (bioMerieux, France); a DNase test carried out on commercially-prepared DNA agar (Biocorp, Poland); a catalase test, using bacterial cultures transferred onto triptic soy agar (Triptic Soy Agar – TSA, bioMerieux, France); and a β-galactosidase activity assay, using the API ZYM commercial kit (bioMerieux, France) according to the producer's recommendations.

Determination of susceptibility of bacteria to selected chemotherapeutic agents

Susceptibility of the isolated bacterial strains to selected antibiotics and sulfonamides was tested using the Kirby-Bauer disk diffusion method on Mueller-Hinton medium (bioMerieux), in accordance with international norms (CLSI 2011). The results were read and interpreted based on the diameter of the zone of inhibition. The strains were designated as resistant (R), of intermediate susceptibility (I) or susceptible (S). The susceptibility profiles of the bacteria were determined for the following agents (OXOID, England): amoxicillin (AML), amoxicillin with clavulanic acid (AMC), cephalexin (CL), chloramphenicol (C), enrofloxacin (ENR), erythromycin (E), flumequine (UB), gentamicin (CN), clindamycin (DA), Linco-Spectin (lincomycin/spectinomycin) (LS), neomycin (N), oxacillin (OX), oxytetracycline (OT), penicillin G (P), streptomycin (S), trimethoprim/sulfamethoxazole (SXT), and tetracycline (TE).

Table 1. Resistance of the *Staphylococcus aureus* strains to antibiotics.

Antibiotic	Number (percentage) of *S. aureus* strains (n=18)	
	resistance	intermediate resistance
Amoxicillin (AML)	8 (44.44%)	0
Amoxicillin with clavulanic acid (AMC)	0	0
Cephalexin (CL)	0	0
Chloramphenicol (C)	0	0
Enrofloxacin (ENR)	5 (27.77%)	1 (5.55%)
Erythromycin (E)	12 (66.66%)	1 (5.55%)
Flumequine (UB)	4 (22.22%)	4 (22.22%)
Gentamicin (CN)	0	0
Clindamycin (DA)	4 (22.22%)	3 (16.66%)
Linco-Spectin (lincomycin/spectinomycin) (LS)	2 (11.11%)	2 (11.11%)
Neomycin (N)	2 (11.11%)	0
Oxacillin (OX)	3 (16.66%)	0
Oxytetracycline (OT)	11 (61.11%)	1 (5.55%)
Penicillin G (P)	9 (50.0%)	0
Streptomycin (S)	3 (16.66%)	0
Trimethoprim/sulfamethoxazole (SXT)	1 (5.55%)	0
Tetracycline (TE)	12 (66.66%)	0

DNA isolation and plasmid profile

All *Staphylococcus aureus* strains were tested for the presence of plasmids. Plasmids were isolated on DNA Gdańsk commercial kits for plasmid DNA isolation using a modification by Schwarz et al. (1990) for staphylococci. Plasmids were separated by electrophoresis in 1% agarose (Sigma Aldrich, USA) at a voltage of 4.5 V/cm; buffer: 1xTAE (Tris-Acetate-EDTA); time: 3 hours. Following electrophoresis, the gels were stained for 15 minutes with ethidium bromide solution (1.0 µg/ml EtBr in 0.5xTBE), and then observed under UV light. The image was registered and analysed using Quantity One® software, version 4.1 (BioRad).

Results

Isolation and identification of bacterial strains

A total of 105 bacterial strains identified as *Staphylococcus* were isolated from the material, of which 18 (17.14%) isolates were of the species *Staphylococcus aureus.* The greatest number of *Staphylococcus aureus* strains (55.5%) were isolated from the shells, while 27.8% of isolates were obtained from the yolks and 16.7% from the whites of the eggs.

The *Staphylococcus aureus* strains were found to exhibit typical biochemical reactions for this species. All of the *Staphylococcus aureus* isolates (18) produced protein A and clumping factor and broke down mannitol. Only eight of the strains synthesized detectable quantities of coagulase in the tube coagulase test. Determination of hemolysis type showed that seven strains exhibited β-hemolysis, while the other 11 exhibited α-hemolysis. Strong activity of DNase and β-galactosidase was observed in nine and five *S. aureus* isolates, respectively. All of the strains tested, both coagulase-positive and coagulase-negative, broke down glucose, fructose, and saccharose, produced alkaline phosphatase, and reduced nitrates to nitrites. None of the strains was found to break down methyl-α-D-glucopyranoside, melibiose, or xylitol.

Determination of susceptibility of bacteria to selected chemotherapeutic agents

All of the 18 *S. aureus* strains tested exhibited resistance to at least one of the antibiotics tested, and some (55.55%) were resistant to five or more of the 17 therapeutic agents. The highest number of strains were resistant to erythromycin (66.66%), tetracycline (66.66%), oxytetracycline (61.11%), penicillin G (50%) and amoxicillin (44.44%). The smallest percentage of strains were found to be resistant to streptomycin and oxacillin (16.66%), neomycin and Linco-Spectin (11.11%), and trimethoprim-potentiated sulfonamides (5.55%). None of the 18 strains exhibited resistance to chloramphenicol, gentamicin, cephalexin, or amoxicillin with clavulanic acid. Detailed data are presented in Table 1.

Table 2. Antibiotic resistance patterns* and presence of plasmids in isolated strains of *Staphylococcus aureus.*

Staphylococcus aureus strain	Antibiotic resistance profile	Number of plasmids	Plasmid size – molecular weight (kb)
ATCC 43300, MRSA	AML, AMC, CL, ENR, E, CN, DA, LS, N, OX, P, S, SXT,	0	–
ATCC 29213, MSSA	AML, C, OX, P, S, TE	1	27.1
SA1	E, ENR, DA, OT, TE	4	2.8, 3.1, 4.1, 5.6
SA2	ENR, UB, LS, OT, TE	11	1.9, 2.2, 2.5, 3.3, 4.1, 4.6, 6.4, 8.4, 9.3, 17.8, 22.1
SA3	OT, TE	7	2.8, 3.1, 4.1, 4.6, 5.6, 6.4, 9.3
SA4	E	4	2.8, 3.1, 3.3,5.6
SA5	AML, OT, OX, P, TE	0	–
SA6	E	1	19.3
SA7	E	1	19.3
SA8	E	2	5.6, 18.5
SA9	AML, E, ENR, P, S, TE	7	2.5, 3.7, 4.1, 7.2, 8.4, 14.2, 18.5
SA10	AML, E, ENR, OT, P, S, TE	4	2.5, 4.6, 5.6, 18.5
SA11	E, OT, TE	4	3.3, 5.6, 7.2, 18.5
SA12	AML, E, ENR, UB, OT, P, S, TE	5	2.5, 3.7, 4.6, 7.2, 15.7
SA13	AML, E, N, OT, P, TE	6	2.5, 3.3, 5.6, 8.4, 16.8, 17.8
SA14	AML, E, DA, OT, P, TE	8	2.5, 3.1, 4.1, 5.6, 7.2, 14.2, 17.3, 19.3
SA15	AML, UB, DA, LS, OT, P, TE	7	3.7, 4.6, 5.6, 6.4, 9.3, 17.8, 20.4
SA16	P, SXT	7	2.5, 3.3, 4.1, 5.6, 6.4, 8.4, 17.3
SA17	OT, OX, TE	9	2.5, 3.3, 4.1, 4.6, 5.6, 6.4, 9.3, 17.8, 20.4
SA18	AML, E, UB, DA, N, OX, P	5	2.5, 3.3, 4.1, 4.6, 5.6

* (AML) – amoxicillin, (AMC) – amoxicillin with clavulanic acid, (CL) – cephalexin, (C) – chloramphenicol, (E) – erythromycin, (ENR) – enrofloxacin, (UB) – flumequine, (CN) – gentamicin, (DA) – clindamycin, (LS) – Linco-Spectin (lincomycin/spectinomycin), (N) – neomycin, (OX) – oxacillin, (OT) – oxytetracycline, (P) – penicillin G, (S) – streptomycin, (STX) – trimethoprim/sulfamethoxazole, (TE) – tetracycline

DNA isolation and plasmid profile

Testing for the presence of plasmid DNA showed that 17 (94.44%) of the *S. aureus* strains contained from 1 to 11 plasmids of varying molecular weight (from 1.9 to 22.1 kb). Most frequently isolated were plasmids of 5.6 kb, noted in as many as 11 (61.11%) of the *S. aureus* strains. Also observed quite frequently were plasmids of 2.5, 4.1, 4.6, and 3.3 kb, in 9 (50%), 8 (44.44%), 7 (38.88%) and 6 (33.33%) strains, respectively. Two of the strains, designated as SA6 and SA7, had one plasmid each with an identical molecular weight of 19.3 kb. Detailed data are presented in Table 2.

Discussion

The present study revealed a fairly high rate of *Staphylococcus* contamination of table eggs. The *Staphylococcus* strains isolated from the material belonged to 12 species, with *Staphylococcus aureus* accounting for 17.14% of isolates. Although most of the *S. aureus* strains were isolated from shells, 27.8% were isolated from yolks and 16.7% from whites. The available literature shows that while these bacteria are isolated from eggs with varying frequency depending on geographical location, they can pose a serious threat to consumer health by inducing food poisoning. In France, for instance, a fairly high percentage (11%) of cases of food poisoning in 1999-2000 resulted from eating eggs and egg products contaminated with staphylococci (Haeghebaert et al. 2002). Analysis of the epidemiological situation of food poisoning and foodborne infections in Poland showed that 25% of food poisoning cases induced by *Staphylococcus aureus* in humans in 2009 were caused by consumption of table eggs (Baumann-Popczyk and Sadkowska-Todys 2011). This was confirmed by Stępień-Pyśniak et al. (2009), who demonstrated that *Staphylococcus aureus* was the second most numerous *Staphylococcus* species

isolated from table chicken eggs, mainly from the yolks and shells.

Another particularly important aspect constituting a serious threat to consumer health is antibiotic resistance of *S. aureus* strains isolated from eggs. *S. aureus* strains occurring in poultry have been shown to be a source of resistance genes for strains isolated from humans, despite the fact that they inhabit different ecosystems (Khan et al. 2000). Plasmids are believed to play a very important role in mediating and transferring resistance to antibacterial drugs in the *Staphylococcus* population (Lacey 1975). They can be vectors of resistance genes, or these genes can be localized in discrete transposable elements of DNA called transposons, which are mobile and can move from one DNA molecule to another. This can lead to the rapid spread of antibiotic resistance in a staphylococcus population, and explains the emergence of multiresistant strains. Most multiresistant strains of *Staphylococcus aureus*, including MRSA, exhibit resistance to erythromycin, tetracycline, and streptomycin, and less often, to gentamicin and chloramphenicol. Almost all MRSA strains also produce β-lactamase (Lacey 1975, Lyon and Skurray 1987).

The results of the present study showed that 55.55% of the *S. aureus* strains tested exhibited resistance to more than five of the therapeutic agents tested. The most frequently observed resistance patterns include lack of susceptibility to erythromycin, tetracycline, oxytetracycline, penicillin G, and amoxicillin. Most of the *S. aureus* strains which showed a high level of resistance to antibiotics had at least four plasmids.

Data from the available literature indicate that the frequency of occurrence of resistance to erythromycin in staphylococci isolated from poultry was found to be substantially higher than in *S. aureus* strains isolated from humans and cattle. The available literature also contains reports that resistance to erythromycin is usually associated with resistance to other macrolides, lincosamides, and type B streptogramin, and is referred to as MLS resistance (Rosdahl et al. 1990)

In the present study, most of the strains (61.53%) that exhibited resistance to erythromycin had plasmids of 5.6 kb. Nevertheless, some of the isolates were found to contain plasmids of 2.5 kb (46.15%) or of 3.3 kb or 4.1 kb (30.76%). Moreover, the plasmid profile analysis of the two strains that exhibited resistance to Linco-Spectin detected plasmids of the same size: 2.5, 3.3, 4.1 and 5.6 kb.

Various mechanisms of antibiotic resistance appear in the *S. aureus* population. In many cases more than one mechanism of resistance to the same group of antibiotics can be distinguished. β-lactamase production by staphylococci is usually of plasmid origin, but in some strains the information is coded in the chromosomal DNA (Gillespie and Skurray 1986).

In the present study, all 8 (44.44%) of the strains that were resistant to amoxicillin were also resistant to penicillin G. The plasmid profile analysis of these strains showed that the plasmids isolated were most often of 2.5 kb (from 7 isolates – 38.88%), 5.6 kb (from 6 – 33.33%), and 4.1 kb and 18.5 kb (from 4 isolates – 22.22%). Moreover, 75% of strains resistant to amoxicillin and penicillin G were also resistant to erythromycin.

A relatively high percentage of the *S. aureus* strains were also found to be resistant to tetracycline (66.66%) and oxytetracycline (61.11%). Data from the literature indicate that resistance to tetracycline in *S. aureus* is usually determined by the presence of small plasmids of 1.3 – 4.6 kb, particularly those of 4.0 to 4.5 kb, which can also contain genes for resistance to chloramphenicol, aminoglycoside antibiotics, cadmium cations, quaternary ammonium compounds, and MLSB (macrolides, lincosamides, and streptogramin B), (Lyon and Skurray 1987). The *S. aureus* strains tested that were resistant to tetracycline usually contained plasmids of 5.6 kb – 75%, 4.6 kb – 58,33%, 2.5kb – 50%, or 4.1, 6.4, or 9.3 kb – 41.66%.

While all the *S. aureus* strains tested in the present study were susceptible to amoxicillin with clavulanic acid, cephalexin, chloramphenicol, and gentamicin, a relatively large percentage were resistant to several of the other agents tested. Moreover, most of the *S. aureus* strains that were resistant to several of the antibiotics had at least four plasmids. This suggests that further research is necessary, including DNA sequencing of isolated plasmids in order to confirm the dependence between antibiotic resistance of *Staphylococcus aureus* strains and the presence of these plasmids.

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