

Characterization of bacteria of the genus *Staphylococcus* isolated from the eggs of Japanese quail (*Coturnix coturnix japonica*)

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Abstract

The study attempted to analyse and characterize bacteria of the genus *Staphylococcus* isolated from the surface and contents of quail eggs, taking into account their phenotypic properties, biochemical reactions, antibiotic sensitivity patterns, and PCR to test for the presence of the *mecA* gene, which is responsible for resistance to methicillin. The study included 45 strains of the genus *Staphylococcus* isolated from the whites, yolks and shells of table quail eggs. The results obtained indicate that a fairly high percentage of the retail quail eggs tested were contaminated with *Staphylococcus* bacteria. Among the species isolated (11 in total), the most frequently occurring strains were of *Staphylococcus hominis* (26.7%), followed by *Staphylococcus aureus* (15.6%), *S. xylosus* and *S. lentus* (13.3% each), while percentages of the other *Staphylococcus* species were under 10%. The *Staphylococcus* strains tested had highly differentiated biochemical and enzymatic properties. As many as 7 biotypes were distinguished among the 7 *S. aureus* isolates, 6 biotypes within the species *S. xylosus* (6 strains tested), 5 biotypes among the 6 strains of *S. lentus*, but only 4 biotypes among the 12 *S. hominis* strains. The antibiotic sensitivity testing showed 15.5% of the strains to be resistant to one or more of the therapeutic agents tested. Moreover, some isolates exhibited intermediate sensitivity to the drugs, particularly to gentamicin (24.4%), neomycin (31.1%), streptomycin (46.7%) and Linco-Spectin (48.9%).

Key words: *Staphylococcus* spp., quail egg contamination, biochemical properties, drug resistance

Introduction

Many *Staphylococcus* species have developed specific biochemical and physiological adaptive mechanisms which enable them to live in particular host organisms. The species that most frequently induces infection in animals and humans is *Staphylococcus aureus*, which is also widespread in nature and can be iso-

lated from the tissues of farm animals – including poultry – that do not exhibit symptoms of illness. Considerable importance is currently given to the role of coagulase-negative staphylococci, which are potentially pathogenic for birds and mammals. Of particular significance in the etiology of infections in humans are the species *Staphylococcus epidermidis*, *Staphylococcus haemolyticus* and *Staphylococcus hominis*. Data

from the literature indicate that it is these species of coagulase-negative staphylococci that have most frequently been isolated from clinical samples in recent years. *Staphylococcus* strains originating in animals can be potentially pathogenic for humans. Their resistance to antibiotics can be zoonotic as well. Staphylococci isolated from animals may differ substantially in terms of physiology and resistance to antibiotics and phages; moreover, they do not all adapt easily to the human organism. Resistant microorganisms are also a source of genes responsible for antibiotic resistance in other bacteria that acquire them.

Increasing attention has been given to the role of poultry and poultry products, including eggs, as a potential source of infections in humans induced by antibiotic-resistant *Staphylococcus* strains (Manie et al. 1998, Abulreesh and Organji 2011). The literature regarding the type of microbial flora on the eggshell and in the egg contents mostly focuses on hatching eggs, because trans-shell contamination of hatching eggs may reduce hatchability (Board and Tranter 1995). However, table eggs are consumed worldwide in various forms and are considered a very nutritious and inexpensive source of protein. Staphylococci constitute an important component of the flora which can be isolated from the surface and contents of table eggs. They have the potential to cause spoilage and enter the food chain causing infection in consumers. The shell can be infected when passing through the vent, but many researchers suggest that contamination mainly occurs within a short period after laying due to contact with contaminated surfaces. It has been hypothesized that bacterial contamination of egg contents could result from the penetration of the shell by bacteria deposited on the surface of the egg after it has been laid (Bahrouz and Al-Jaff 2007). The information available, mainly based on analysis of the microfloral composition of hens' eggs, shows that Gram-positive bacteria, including staphylococci, predominate on the shell surface, probably because they are better adapted to development in a dry environment, and because the dust, soil or faeces in poultry houses are contaminated with these bacteria. Little is known about the role of eggs from other poultry species in spreading *Staphylococcus* infections.

In view of the growing popularity of Japanese quail (*Coturnix japonica*) breeding and of retail sale of quail eggs, the present study attempted to analyse the composition and characteristics of bacteria of the genus *Staphylococcus* isolated from the shell surface and the contents of quail eggs, taking into account their phenotypic properties, biochemical reactions, antibiotic sensitivity patterns, and selected genotypic characteristics.

Materials and Methods

Material

The study included 70 table quail eggs purchased in shops in the city of Lublin. The eggs were clean, with no cracks or visible flaws in the shells, and in each case the expiry date on the package indicated that the eggs were fresh.

The study analysed 45 strains of the genus *Staphylococcus* isolated from the whites, yolks and shells of table quail eggs, identified as follows: *Staphylococcus aureus* - 7 strains, *Staphylococcus hominis* - 12 strains, *Staphylococcus xylosum* - 6 strains, *Staphylococcus lentus* - 6 strains, *Staphylococcus sciuri* - 4 strains, *Staphylococcus epidermidis* - 3 strains, *Staphylococcus caprae* - 2 strains, *Staphylococcus hyicus* - 2 strains, and one strain each of *Staphylococcus cohnii*, *Staphylococcus simulans*, and *Staphylococcus auricularis*. Two reference strains, methicillin-resistant *Staphylococcus aureus* (ATCC 43300, MRSA) and methicillin-sensitive *Staphylococcus aureus* (ATCC 29213, MSSA), obtained from the Central Laboratory of Sera and Vaccines in Warsaw, 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, and 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 tryptic soy agar (Tryptic 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 the sensitivity of the bacteria to selected chemotherapeutic agents

Sensitivity of the isolated bacterial strains to selected antibiotics and sulfonamides was tested using the Kirby-Bauer disk diffusion method on Mueller-Hinton medium (BioMerieux, France), in accordance with accepted international norms (CLSI 2011). The results were read and interpreted based on the diameter of the zone of inhibition, with the strains designated as resistant (R), of intermediate sensitivity (I) or sensitive (S). The sensitivity profiles of the bacteria were determined for the following agents (OXOID, England): amoxicillin (AMX), amoxicillin with clavulanic acid (AMC), cephalexin (CL), doxycycline (D), enrofloxacin (ENR), norfloxacin (NOR), gentamicin (CN), Linco-Spectin (lincomycin/spectinomycin) (LS), neomycin (N), oxytetracycline (OT), streptomycin (S) and trimethoprim/sulfamethoxazole (SXT).

The reference system comprised the results of the drug sensitivity tests for the reference strains: methicillin-resistant (ATCC 43300, MRSA) and methicillin-sensitive (ATCC 29213, MSSA) *Staphylococcus aureus*.

Evaluation of the bacterial strains' genetically-conditioned resistance to methicillin

All of the isolated *Staphylococcus* strains were tested by PCR for the presence of the *mecA* gene, which conditions resistance to methicillin (Brakstad et al. 1993). Two reference strains, methicillin-sensitive *Staphylococcus aureus* (ATCC 29213) and methicillin-resistant *Staphylococcus aureus* (ATCC 43300), were used as the control. Primers complementary to the conservative region in the *mecA* gene, 533 bp in length, were used for the amplification. The primers were synthesized at DNA-Gdańsk. Amplification 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). The PCR result was considered to be positive when single DNA bands whose size was determined by the location of the corresponding primer pairs in the genome were present in the gel.

Results

Isolation and identification of bacterial strains

A total of 45 *Staphylococcus* strains from 11 species were isolated from the material, including 12 strains of *Staphylococcus hominis* (26.7%), 7 of *S. aureus* (15.6%), 6 of *S. xylosum* (13.3%), 6 of *S. lentus* (13.3%), 4 of *S. sciuri* (8.9%), 3 of *S. epidermidis* (6.7%), 2 of *S. caprae* (4.4%), 2 of *S. hyicus* (4.4%), 1 of *S. cohnii* (2.2%), 1 of *S. simulans* (2.2%), and 1 of *S. auricularis* (2.2%). The greatest number of strains (77.8%) were isolated from shells, while 15.6% of isolates were obtained from yolks and 6.6% from the whites of the eggs. The types of bacteria isolated from different parts of the egg are presented in Fig. 1.

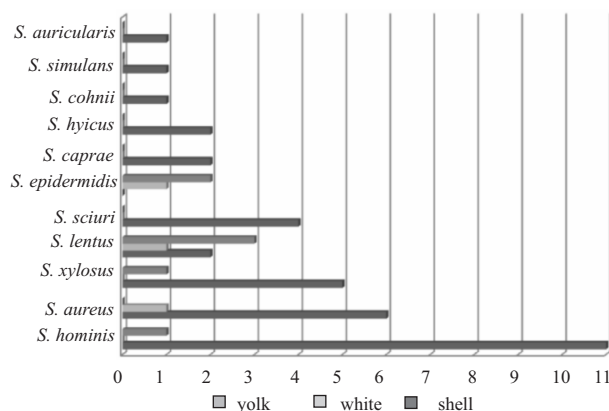


Fig. 1. Type of *Staphylococcus* bacteria isolated from whites, yolks and shells.

Drug sensitivity of the bacterial strains

Tests of the sensitivity of the 45 *Staphylococcus* strains to antibiotics and chemotherapeutic agents found 7 strains (15.5%) that were resistant to some of the drugs applied. These were *S. hominis* – 1 strain resistant to gentamicin and 2 strains resistant to streptomycin, *S. epidermidis* – 1 strain resistant to oxytetracycline, *S. hyicus* – 1 strain resistant to gentamicin and streptomycin, *S. cohnii* – 1 strain resistant to Linco-Spectin and *S. simulans* – 1 strain resistant to doxycycline, gentamicin, norfloxacin, streptomycin and trimethoprim/sulfamethoxazole. None of the strains was found to be resistant in *in vitro* conditions to cephalexin (CL), enrofloxacin (ENR), amoxicillin with clavulanic acid (AMC), amoxicillin (AML) or neomycin (N). However, a certain percentage of isolates was observed to have intermediate sensitivity to these agents – 4.4% of strains exhibited intermediate sensitivity to cephalexin and enrofloxacin, 8.9% to amoxicillin with clavulanic acid, 13.3% to amoxicillin, and 31.1% to neomycin. Detailed data are presented in Fig. 2.

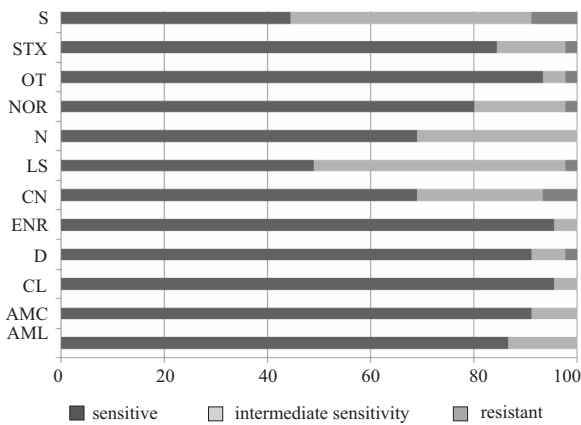


Fig. 2. Sensitivity of isolated staphylococci to chemotherapeutic agents*.

* (AML) – amoxicillin, (AMC) – amoxicillin with clavulanic acid, (CL) – cephalexin, (D) – doxycycline (ENR) – enrofloxacin, (NOR) – norfloxacin, (CN) – gentamicin, (LS) – Linco-Spectin (lincomycin/spectinomycin), (N) – neomycin, (OT) – oxytetracycline, (S) – streptomycin, (SXT) – trimethoprim/sulfamethoxazole.

Biochemical and enzymatic properties of the strains

The *Staphylococcus* strains tested had highly varied biochemical and enzymatic properties. As many as 7 biotypes were distinguished among the 7 isolates of *S. aureus*, 6 biotypes within the species *S. xylosus* (6 strains tested), 5 biotypes among the 6 strains of *S. lentus*, but only 4 among the 12 strains of *S. hominis*. Detailed data are presented in Table 1.

Biochemical identification of all the *Staphylococcus aureus* strains showed that they belonged to *S. aureus* subsp. *aureus* and that their biochemical characteristics were highly homologous to the reference strains.

Genetically conditioned resistance to methicillin

The results of PCR used to detect the methicillin resistance gene (*mecA*), which determines the production of a protein in the bacterial cell wall with low affinity for beta-lactam antibiotics, did not confirm the presence of a specific product in the form of a 533 bp band in any of the samples tested.

Discussion

The results obtained indicate that a fairly high percentage of the retail quail eggs tested were contaminated with *Staphylococcus* bacteria. The substantial species diversity of the bacteria isolated is worth

mentioning. While *Staphylococcus aureus* is the most frequent cause of infections in poultry, other *Staphylococcus* species, though far less often described by other authors, cannot be treated exclusively as commensals in birds. They are often a direct infectious agent for both people and animals (Gill 1983). Reports can be found in the literature of the frequent occurrence of coagulase-negative staphylococci, not only in the contents and on the shells of eggs but also in the tissues of birds (Wieliczko et al. 2002, Stępień-Pyśniak et al. 2009).

While most of the staphylococci were found on the shells of the eggs, a certain percentage of strains of the species *S. lentus*, *S. xylosus*, and *S. hominis* were also noted in the yolk and white. The *S. epidermidis* strains, which were isolated exclusively from the white and yolk, were an exception. *Staphylococcus* bacteria are isolated from hens' eggs with varying frequency. In a study by Stępień-Pyśniak et al. (2009), the species most frequently isolated from the environment of the egg white were *S. warneri* and *S. epidermidis*, while the species isolated most frequently from the yolk was *S. aureus*, regardless of the source of the eggs. In the present study, the most frequently isolated species was the coagulase-negative *Staphylococcus hominis*. Analysis of the biochemical properties of the 12 strains of this species, using API tests, found differences in the breakdown of only 4 substrates. The biochemical patterns of 5 of the 12 strains were 100% similar. In the case of the *Staphylococcus aureus* strains, a certain percentage of isolates had an atypical biochemical profile. For this reason additional tests were conducted, in which clumping factor production, the presence of surface protein A, and strong DNase activity were found in 100%, 57% and 71%, respectively, of the *S. aureus* strains. Only two *S. aureus* strains (28.6%) and one *S. hyicus* strain (50%) produced coagulase in the tube coagulase test. All of the other isolates tested were coagulase-negative. Coagulase production combined with clumping factor synthesis usually occurs only in *Staphylococcus aureus* subsp. *aureus* and in some strains of *S. intermedius* (Młynarczyk et al. 1997). Some *Staphylococcus* species exhibit highly varied sensitivity to the effects of lysozyme contained in egg white. Thompson and Khorazo (1935) observed that staphylococci that produce an orange pigment, ferment mannitol and produce coagulase are more resistant to lysozyme than strains lacking these biochemical characteristics. This is partially confirmed by the results of the present study, as the only *S. aureus* strain isolated from egg white exhibited all of these traits.

The antibiotic sensitivity tests showed that 15.5% of the strains were resistant to one or more of the therapeutic agents applied. The highest percentages

Table 1. Biochemical properties of the isolated *Staphylococcus* strains.

Property	<i>S. aureus</i> n=7	<i>S. hominis</i> n=12	<i>S. xylosus</i> n=6	<i>S. lentus</i> n=6	<i>S. sciuri</i> n=4	<i>S. epidermidis</i> n=3	<i>S. caprae</i> n=2	<i>S. hyicus</i> n=2	<i>S. cohnii</i> n=1	<i>S. simulans</i> n=1	<i>S. auricularis</i> n=1
Pigment production	2	10	1	3	1	0	1	0	0	0	0
Koagulase	2	0	0	0	0	0	0	1	0	0	0
β hemolysis	2	0	2	1	1	0	0	0	0	0	0
α hemolysis	5	0	0	0	0	0	0	0	0	0	0
DNase	5	2	1	2	1	0	1	2	0	1	0
Catalase	7	12	6	6	4	3	2	2	1	1	1
Clumping factor	7	0	0	0	0	0	0	0	0	0	0
Protein A	4	0	0	0	0	0	0	0	0	0	0
Acid production from:											
– glucose	7	12	6	6	4	3	2	2	1	1	1
– fructose	7	12	6	6	4	3	2	2	2	1	1
– mannose	4	2	6	6	4	3	0	2	1	0	0
– maltose	6	12	6	6	4	3	1	0	0	0	1
– lactose	7	0	6	6	1	3	0	0	1	1	0
– trehalose	7	12	6	6	4	0	2	2	0	1	1
– mannitol	4	0	6	6	4	0	0	0	1	0	0
– raffinose	1	0	3	6	0	0	0	0	0	0	0
– saccharose	7	12	5	6	4	3	2	2	1	1	1
– NAG*	6	0	6	6	1	0	0	0	0	1	0
– MDG**	0	0	6	1	1	0	0	0	0	0	0
– melibiose	0	0	2	5	0	0	0	0	0	0	0
– xylitol	0	0	0	1	0	0	0	0	0	0	0
– xylose	1	0	6	6	1	0	0	0	0	0	0
Production of:											
– urease	5	5	4	0	0	3	0	2	0	1	0
– ADH***	7	3	0	0	0	3	0	2	0	1	0
– β -galactosidase	3	3	0	5	1	1	1	0	0	0	1
– alkaline phosphatase	7	12	6	6	4	3	2	2	1	1	1
– nitrate reduction	7	12	5	5	3	3	2	2	0	1	1
– acetoin****	5	10	2	3	1	3	0	0	0	0	0

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

of strains were resistant to streptomycin (8.9%) and gentamicin (6.7%). According to the literature, resistance both to streptomycin and to gentamicin in *Staphylococcus* bacteria can result from the presence of genes localized in plasmid DNA, which facilitates the transfer of resistance determinants to other strains which also occur in humans (Grinsted and Lacey 1973, Lyon et al. 1987). The strains exhibiting resistance to more than one of the antibiotics were

Staphylococcus simulans and *Staphylococcus hyicus*, which were resistant to five and two of the antibiotics, respectively. A small percentage (2.2%) of the staphylococci tested were resistant to synthetic chemotherapeutic agents of the fluoroquinolone group, which are widely used to treat bacterial infections in poultry. Resistance to this group of antibiotics is observed with increasing frequency among *Staphylococcus* bacteria (Aarestrup et al. 2000, Khan

et al. 2000). In a study by Wieliczko et al. (2002), evaluation of the antibiotic sensitivity of *Staphylococcus* strains isolated from poultry showed that in *in vitro* conditions the strains were most sensitive to amoxicillin and amoxicillin with clavulanic acid. The results of the present study showed that none of the bacteria tested were resistant to amoxicillin and amoxicillin with clavulanic acid in *in vitro* conditions, but a certain percentage of strains (13.3% and 8.9%) showed intermediate sensitivity to these antibiotics.

Observations made in recent years have demonstrated the role of staphylococci as an important indicator of the increase in, and emergence of, resistance to therapeutic agents used against micro-organisms. The confirmed presence of the same resistance genes in staphylococci isolated from humans and animals, as well as the possibility of transference of genetic determinants between different species of bacteria, suggest that *Staphylococcus* bacteria, particularly CNS strains, are a reservoir of genes of antibiotic resistance in the natural environment (Aarestrup et al. 2000, Khan et al. 2000). Many authors also emphasize the role of poultry and poultry products as a significant source of outbreaks of food poisoning caused by *Staphylococcus* bacteria (Miwa et al. 2001, Przybylska 2001).

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