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Detection of antibiotic resistance and classical enterotoxin genes in coagulase -negative staphylococci isolated from poultry in Poland

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Abstract

Introduction: The study sought to characterise antimicrobial resistance among coagulase-negative *Staphylococcus* (CNS) species recovered from broiler chickens and turkeys in Poland including the presence of 12 antimicrobial resistance genes and five classical genes of staphylococcal enterotoxins. **Material and Methods:** A panel of 11 antimicrobial disks evaluated the phenotypic sensitivity of the tested strains to antibiotics. Five multiplex PCR assays were performed using primer pairs for specific detection of antibiotic resistance genes and staphylococcal enterotoxin A to E genes. **Results:** Selected antimicrobial agent susceptibility testing revealed 100% of such in *in vitro* conditions to cefoxitin among strains of *Staphylococcus sciuri* and *S. chromogenes*. The *bla*Z (for ß-lactam) and *mec*A (for methicillin resistance) genes were in 58.3% and 27.5% of strains, respectively. Among genes resistant to tetracyclines, *tet*K was most frequent. Fewer (CNS) strains showed genes resistant to macrolides, lincosamides, and florfenicol/chloramphenicol. Multiplex PCR for classical enterotoxins (A-E) detected the *see* gene in two *S. hominis* strains, while the *seb* gene producing enterotoxin B was found in one strain of *S. epidermidis*. **Conclusion:** CNS strains of *Staphylococcus* isolated from poultry were either phenotypically or genotypically multidrug resistant. Testing for the presence of the five classical enterotoxin genes showed that CNS strains, as in the case of *S. aureus* strains, can be a source of food intoxications.

Keywords: poultry, coagulase-negative *Staphylococcus*, methicillin-resistant *Staphylococcus*, antimicrobial resistance genes, enterotoxigenicity.

Introduction

The genus *Staphylococcus* consists of 51 species and 27 sub-species (www.bacterio. net/staphylococcus. html). The coagulase-positive *S. aureus* causes infections in humans and animals, and is consequently the most important species in this genus and one of the causes of food intoxication (1, 3, 5, 18, 24). Other *Staphylococcus* species termed coagulase-negative staphylococci (CNS) have gained in importance as they have been implicated in a variety of opportunistic infections in humans and animals (21, 26). Until recently, CNS were considered non-pathogenic members of the genus and thus were of little interest to scientists, but following their implication in infections in humans and animals, research interest in CNS has increased over the past decade (29). Coagulase-negative staphylococci, including many species such as *S. gallinarum*, *S. arlettae*, *S. chromogenes*, *S. xylosus*, and *S. epidermidis*, have commonly been isolated from the nares and skin of chickens (24, 29). Although CNS in chickens have generally been accepted as harmless inhabitants, it has gradually become clear that they manifest pathogenicity under suitable conditions. The

CNS *S. sciuri*, *S. simulans*, and *S. epidermidis* have been isolated from cases of dermatitis, tendonitis, and endocarditis in chickens (26). CNS can also be opportunistic pathogens in humans, when they are often associated with medical devices, which the frequent causation of nosocomial infections by *S. epidermidis* bears out (21). The pathogenesis of CNS species relies on factors required for their commensal mode of life and one such factor increasing the importance of these microorganisms in the pathology of mammals and birds is their resistance to numerous antimicrobial agents (29). Slaughter poultry has been identified as one of the most important vehicles for antimicrobial-resistant foodborne pathogens and antimicrobial resistance genes (31). Detailed analysis of the resistance genes present in livestock-associated staphylococci has revealed a wide variety of them. These mainly include genes which are known to be commonly present in staphylococci of human and animal origin, such as the β-lactamase gene *blaZ*, methicillin resistance gene *mec*A, tetracycline resistance genes *tet*(K), *tet*(L), *tet*(M), and *tet*(O), macrolide-lincosamide-streptogramin B (MLSB) resistance genes *erm*(A) and *erm*(B), gene of inducible resistance to erythromycin *msr*A/B, *aac (6')-Ie aph (2")-Ia* gene of aminoglycoside modifying enzymes, and florfenicol/ chloramphenicol resistance gene (*cfr*) (31). Resistance of *Staphylococcus* to methicillin is currently a global problem. The mechanisms of methicillin resistance in CNS are identical to those reported for *S. aureus*; however, *mecA* gene-mediated resistance is frequently expressed at lower levels than in methicillin-resistant *S. aureus* (MRSA), which makes it difficult to detect (31).

Although research interest in CNS has increased in recent years, there is very little data on their prevalence and resistance profiles in poultry production. Therefore, the objectives of this study were to characterise the diversity of CNS *Staphylococcus* recovered from poultry in western Poland and to assess the isolates as potential dispersion vectors for the spread of antimicrobial resistance genes and genes encoding enterotoxicity.

Material and Methods

Sample collection. The study was conducted on material derived from broiler chickens and turkeys on farms located in central-western Poland between November 2016 and December 2017. During this time, samples from 137 flocks were collected. From each flock, randomly selected birds showing clinical signs of disease were examined and sampled to provide 3 to 5 specimens from the affected organs of each bird. A total of 435 samples from broilers and 112 samples from turkeys were collected, comprising material from the heart, liver, tarsal joints, and bone marrow of birds aged 1 day to 6 weeks (chickens) or 20 weeks (turkeys). The birds from which samples were taken showed increased mortality, dermatitis, cellulitis, lameness, arthritis, decreased weight gain, omphalitis, or yolk sac infections. The size of the flocks from which the samples were collected ranged from 8,000 to 44,000 birds.

The material collected was plated on a blood agar medium (Blood LAB-AGAR, Biocorp, Poland) and Chapman selective medium (Mannitol Salt LAB-AGAR, Biocorp, Poland) and incubated under aerobic conditions at 37°C for 24–48 h, depending on the rate of growth of the bacteria. Single colonies were transferred to blood agar to isolate pure bacterial cultures, and a preliminary bacteriological characterisation was made of the isolated microbiota, involving Gram-staining, cell morphology and motility, examination under a microscope, and determination of the type of haemolysis. Quantitative measurement of the colonies was not performed. Isolated bacteria were stored for further testing at −85°C in 50% (v/v) glycerol in brain heart infusion broth (BHI, Sigma, Germany).

Identification of bacterial strains. Identification of all *Staphylococcus* strains was carried out with MALDI-TOF mass spectrometry using the IVD MALDI Biotyper (Bruker Daltonik, Germany), as described by Marek *et al.* (15).

Bacterial DNA extraction. Total DNA was extracted from strains inoculated individually on blood agar and incubated at 37°C/24 h. The Novabeads Bacterial DNA kit (Novazym, Poland) was used for DNA extraction according to the manufacturer's protocol.

Antimicrobial susceptibility testing. The phenotypic antimicrobial sensitivity response was evaluated using a panel of 11 antimicrobial discs which belonged to seven classes of antimicrobial agents (13). Disc diffusion tests were performed for each of the 127 isolates in their species groups, identified to be *S. cohnii*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. simulans*, *S. saprophyticus*, *S. lentus*, *S. xylosus*, *S. sciuri*, and *S. chromogenes* using the method recommended by the CLSI (4). The drug-susceptibility profiles for the bacteria were determined for the following agents, supplied as discs (Oxoid, UK): amoxicillin 25 µg (AML25), amoxicillin + clavulanic acid 20 + 10 µg (AMC30), ampicillin 10 µg (AMP10), penicillin G 10 units (P10), cefoxitin 30 µg (FOX30), clindamycin 2 µg (DA2), chloramphenicol 30 µg (C30), erythromycin 15 µg (E15), tetracycline 30 µg (TE30), gentamycin 10 µg (CN10), and trimethoprim sulphamethoxazole 1:19, 25 µg (SXT25). For quality control, *S. aureus* ATCC 25923, *S. aureus* ATCC43300, and *S. aureus* ATCC 29213 were used in the disc diffusion tests.

Table 1. Nucleotide sequences and sizes of PCR products of antibiotic resistance genes and enterotoxins (A–E)

Primer*	Oligonucleotide sequence (5'-3')**	Gene	Size of amplified product (bp)	Control strain	References
MEC-1 MEC-2	AAAATCGATGGTAAAGGTTGGC AGTTCTGGCACTACCGGATTTGC	*mec*A	533	*S. aureus* ATCC43300	(17)
BLAZ-1 BLAZ-2	ACTTCAACACCTGCTGCTTTC TAGGTTCAGATTGGCCCTTAG	*blaZ*	240	*S. aureus* ATCCBAA-2312	(7)
TETM-1 TETM-2	GAACTCGAACAAGAGGAAAGC ATGGAAGCCCAGAAAGG T	*tet*M	406	*S. pyogenes* 7020	
TETK-1 TETK-2	TCGATAGGAACAGCAGTA CAGCAGATCCTACTCCTT	*tet*K	155	*E. faecium* ET18	(13)
TETL-1 TETL-1	TGGTGGAATGATAGCCCATT CAGGAATGACAGCACGCTAA	*tet*L	229		
TETO-1 TETO-2	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	*tet*O	515	*E. faecalis* JH2-2TET	
ERMA-1 ERMA-2	CCCGAAAAATACGCAAAATTTCAT CCCTGTTTACCCATTTATAAACG	*erm*A	590	*S. haemolyticus* 955	(13, 14)
ERMB-1 ERMB-2	TGGTATTCCAAATGCGTAATG CTGTGGTATGGCGGGTAAGT	*erm*B	745	*E. faecium* PE1	
MSRA/B-1 MSRA/B-2	GCAAATGGTGTAGGTAAGACAACT ATCATGTGATGTAAACAAAAT	*msr*A/B	399	*S. aureus* RN4220	(26)
ACC-1 ACC-2	CAGGAATTTATCGAAAATGGTAGAAAAG CACAATCGACTAAAGAGTACCAATC	*acc(6')-le-aph(2")-la*	348	*E. faecium* M48	(27, 28)
CFR-1 CFR-2	TGAAGTATAAAGCAGGTTGGGAGTCA ACCATATAATTGACCACAAGCAGC	*cfr*	746		(11)
ESA1 ESA2	ACGATCAATTTTTACAGC TGCATGTTTTCAGAGTTAATC	*sea*	144	*S. aureus* FRI913	(1)
ESB1 ESB2	GAATGATATTAATTCGCATC TCTTTGTCGTAAGATAAACTTC	*seb*	416	ATCC13566	(5)
ESC1 ESC2	GACATAAAAGCTAGGAATTT AAATCGGATTAACATTATCCA	*sec*	257	*S. aureus* FRI913	(1)
ESD1 ESD2	TTACTAGTTTGGTAATATCTCCTT CCACCATAACAATTAATGC	*sed*	334	*S. aureus* FRI151m	(3)
ESE1 ESE2	ATAGATAAAGTTAAAACAAGCAA TAACTTACCGTGGACCC	*see*	170	*S. aureus* FRI913	(3)

* The sets of primers were synthesised by Genomed S.A., Poland.
** The concentration of primers was 0.04 μmol

Multiplex PCR for antibiotic resistance genes. The PCR primers for the *blaZ*, *mec*A, *aac(6')-aph(2")*, *erm*A, *erm*B, *msr*A/B, *tet*M, *tet*K, *tet*L, *tet*O, and *cfr* antibiotic resistance genes are listed in Table 1. Four different multiplex PCR assays were used, including all of the primer pairs for specific detection of antibiotic resistance genes. Multiplex PCR amplifications were performed according to Malhota-Kumar *et al.* (14), Toomey *et al.* (27), Vakulenko *et al.* (28), and Garofalo *et al.* (7). An internal control was integrated into every PCR to verify the efficiency of the amplification and to ensure that there was no significant PCR inhibition.

Prevalence of toxin genes in *Staphylococcus* isolates. Strains were assayed in a multiplex PCR for detection of genes to staphylococcal enterotoxins A to E (*sea*, *seb*, *sec*, *sed*, and *see*) using five pairs of primers (Table 1). The conditions for multiplex PCR were previously described by Bystroń *et al.* (3).

Results

Bacterial strains. A total of 237 bacterial strains belonging to the genus *Staphylococcus* and representing 23 species were isolated from test material. The percentages of strains belonging to each species were as follows: *S. aureus* 30.0%, *S. cohnii* 14%, *S. saprophyticus* 8.4%, *S. epidermidis* 7.2%, *S. xylosus* 6.3%, *S. pseudintermedius* 5.1%, *S. sciuri* 4.2%, *S. lentus* 4.2%, *S. chromogenes* 3.4%, *S. arlettae* 3.4%, *S. equorum* 3%, *S. simulans* 2.1%, *S. hominis* 1.7%, *S. haemolyticus* 1.7%, *S. schleiferi* subsp. *schleiferi* 1.3%, *S. intermedius* 0.8%, *S. warneri* 0.8%, *S. alactolyticus* 0.4%, *S. capitis* 0.4%, *S. condimenti* 0.4%, *S. felis* 0.4%, *S. vitulinus* 0.4%, and *S. pasteuri* 0.4%.

Staphylococcus strains belonging to 10 species were selected for further analysis. These were *S. cohnii* (n = 33), *S. saprophyticus* (n = 20), *S. epidermidis* (n = 17), *S. xylosus* (n = 15), *S. lentus* (n = 10), *S. sciuri* (n = 10), *S. chromogenes* (n = 9), *S. simulans* (n = 5), *S. haemolyticus* (n = 4), and *S. hominis* (n = 4).

Phenotypic susceptibility of the isolated bacteria to selected antimicrobial agents. The susceptibility testing of the isolated strains to 11 selected antimicrobial agents showed 100% susceptibility in *in vitro* conditions to amoxicillin + clavulanic acid of 20 strains of *S. saprophyticus* and 10 strains of *S. sciuri*. Cefoxitin susceptibility of 100% was observed in 10 strains of *S. sciuri* and 9 strains of *S. chromogenes*. A relatively high percentage of CNS strains were found to be susceptible to chloramphenicol

(96.9%), clindamycin (94.5%), trimethoprim-sulphamethoxazole (92.9%), erythromycin (85.8%), and amoxicillin (78.7%). Considerably more isolates exhibited resistance to gentamycin (25.2%). Almost half of the CNS strains were resistant to ampicillin (49.6%), and over half to other antimicrobial agents, with 54.3% of strains resistant to penicillin G and 59.1% resistant to tetracycline. Detailed data are presented in Table 2. The antimicrobial resistance patterns of the isolates are shown in Table 3.

Antimicrobial resistance genes. All isolates were investigated for the presence of genes encoding resistance to methicillin, beta-lactamase, tetracycline, macrolide-lincosamide-streptogramin B (MLS_B), macrolides and streptogramin B (MS_B type resistance), aminoglycoside, and florfenicol/chloramphenicol. The results are summarised in Table 4.

Of the 127 CNS strains, 35 had the *mec*A gene: 9/17 *S. epidermidis*, 6/33 *S. cohnii*, 5/15 *S. xylosus*, 4/10 *S. sciuri*, 3/9 *S. chromogenes*, 3/4 *S. haemolyticus*, 2/4 *S. hominis*, 1/20 *S. saprophyticus*, 1/10 *S. lentus*, and 1/5 *S. simulans*. The presence of the *bla*Z gene determining resistance to β-lactam antibiotics was demonstrated in 74 strains of coagulase-negative staphylococci. This gene was detected in 100% of strains belonging to the species *S. hominis*, 88.2% of *S. epidermidis*, 78.8% of *S. cohnii*, 75% of *S. haemolyticus*, and 60% of *S. lentus*. However, fewer than half of the strains belonging to other species revealed the presence of the *bla*Z gene. Tetracycline resistance in the results was indicated most frequently by the genes *tet*K (in 55% of *S. saprophyticus* and 53.3% of *S. xylosus*) and *tet*L (in 77.8% of *S. chromogenes* and 75% of *S. haemolyticus*). The presence of the *tet*M gene was demonstrated in 60% strains of *S. lentus* and 60% of *S. simulans*. The presence of the *tet*O gene was noted in 2 out of 9 isolates of *S. chromogenes*, 2 out of 10 *S. sciuri* isolates, and in 1 out of 17 *S. epidermidis* isolates. In the case of the two erythromycin ribosomal methylase genes tested, 4.72% strains of CNS contained *erm*A (2/33 *S. cohnii*, 1/4 *S. haemolyticus*, 1/10 *S. sciuri* and 2/9 *S. chromogenes*), and 4.72% contained the *erm*B gene (1/17 *S. epidermidis,* 3/10 *S. lentus*, and 2/15 *S. xylosus*). The *msr*A/B gene encoding an ATP-dependent efflux pump was shown in 7.9% of the CNS strains (3/33 strains of *S. cohnii,* 3/17 *S. epidermidis,* 1/4 *S. hominis,* 1/15 *S. xylosus,* and 2/10 *S. sciuri*). The *aac(6')-Ie-aph(2")-Ia* gene was determined present in 11 isolates (1/33 *S. cohnii*, 6/17 *S. epidermidis*, 1/20 *S. saprophyticus*, 1/10 *S. sciuri*, and 2/9 *S. chromogenes*). The genomes of three CNS strains contained the florfenicol/chloramphenicol resistance gene (*cfr*), those strains belonging to the species *S. cohnii*, *S. epidermidis*, and *S. saprophyticus*.

Prevalence of toxin genes in *Staphylococcus* isolates. The results of a multiplex PCR for the five classical enterotoxins A–E showed that the genome of two *S. hominis* strains contained the gene *see*, responsible for the production of enterotoxin E. The gene responsible for the production of enterotoxin B *(seb*) was found in one strain of *S. epidermidis*. None of the CNS strains had genes responsible for the production of enterotoxins A, C or D.

Table 2. Phenotypic* antimicrobial resistance of *Staphylococcus* strains isolated from broiler chickens and turkeys

Antibiotic	*S. epidermidis*		*S. hominis*		*S. saprophyticus*		*S. lentus*		*S. xylosus*		*S. haemolyticus*		*S. sciuri*		*S. simulans*		*S. chromogenes*		*S. cohnii*		Total[2]	
	n = 17		n = 4		n = 20		n = 10		n = 15		n = 4		n = 10		n = 5		n = 9		n = 33		n = 127	
	R[1]	I[1]	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	Number	%
Amoxicillin	3	2	3	-	2	-	1	1	3	-	2	1	1	-	-	1	2	1	3	1	27	21.3
Amoxicillin + clavulanic acid	2	-	1	-	-	-	1	-	2	-	1	-	-	-	-	1	1	-	1	-	10	7.9
Ampicillin	9	-	4	-	8	1	6	-	7	-	2	2	3	-	1	1	3	-	16	-	63	49.6
Penicillin G	10	1	4	-	8	-	7	-	9	-	4	-	2	-	3	1	4	-	15	1	69	54.3
Cefoxitin	7	-	1	-	1	-	2	-	3	-	1	-	-	-	1	-	-	-	5	-	21	16.5
Clindamycin	2	-	-	1	-	-	1	-	1	-	1	-	-	-	-	-	-	-	1	-	7	5.5
Chloramphenicol	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	4	3.1
Erythromycin	4	-	1	-	-	-	3	-	2	-	1	-	2	-	-	-	-	-	5	-	18	14.1
Gentamycin	5	1	1	-	4	-	4	1	7	-	-	-	4	-	-	-	2	-	3	-	32	25.2
Tetracycline	8	-	2	-	15	4	6	1	9	-	2	2	3	-	3	-	6	-	13	1	75	59.1
Trimethoprim - sulphamethoxazole	2	-	-	-	3	-	1	-	-	-	-	-	-	-	-	-	1	-	2	-	9	7.1

* The susceptibility of 11 antibiotics was tested using a standard disc diffusion method on Mueller–Hinton agar plates

[1] R - resistant, I - intermediate

[2] The resistance rate was calculated as the number of intermediate and resistant isolates divided by the total number of isolates

Table 3. Multidrug resistance profiles of CNS from broiler chickens and turkeys

Staphylococcus spp.	Phenotypic resistance combination pattern[1,2]	n = number of classes antimicrobial agents[3]	number of strains
S. cohnii	P, AMP	1	2
	P, AMP, TE	2	7
	P, AMP, TE, SXT	3	2
	P, AMP, TE, AML, FOX, E	3	2
	P, AMP, TE, AML, FOX, E, CN, C	5	2
	P, AMP, TE, AMC, FOX, E, CN, DA	5	1
S. saprophyticus	TE	1	10
	TE, AMP	2	1
	TE, P, AMP	2	3
	TE, P, AMP, AML, FOX	2	1
	TE, P, AMP, CN, SXT	4	3
	TE, P, AMP, CN, AML, C	4	1
S. epidermidis	P	1	2
	P, AMP, CN	2	1
	P, AMP, TE	2	1
	P, AMP, FOX, TE, SXT	3	1
	P, AMP, FOX, TE, SXT, C	4	1
	P, AMP, FOX, TE, CN, AML	3	1
	P, AMP, FOX, TE, CN, AML, E, DA	5	2
	P, AMP, FOX, TE, CN, AML, E, AMC	4	2
S. xylosus	P, TE	2	2
	P, AMP, TE, CN	3	2
	P, AMP, TE, CN, E	4	2
	P, AMP, TE,CN, AMC, AML, FOX	3	2
	P, AMP, TE, CN, AML, FOX, DA	4	1
S. sciuri	E, CN	2	2
	AMP, TE, CN	3	1
	AMP, TE, P	2	1
	AMP, TE, P, CN, AML	3	1
S. lentus	P, TE	2	1
	P, AMP, TE, SXT	3	1
	P, AMP, TE, CN, E	4	3
	P, AMP, TE, CN, AML, FOX	3	1
	P, AMP, TE, CN, AMC, AML, FOX, DA	4	1
S. chromogenes	TE	1	1
	TE, P, CN	3	2
	TE, AMP, AML	2	1
	TE, P, AMP, AML, SXT	3	1
	TE, P, AMP, AML, AMC	3	1
S. simulans	P, TE	2	2
	P, AMP, TE	2	1
	P, AMP, AML, AMC, FOX	2	1
S. hominis	P, AMP, CN, E	3	1
	P, AMP, AML, TE	2	2
	P, AMP, AML, AMC, FOX, DA	2	1
S. haemolyticus	P, AMP, AML, TE	2	3
	P, AMP, AMC, DA, E, FOX, TE	4	1

[1]AMC – amoxicillin + clavulanic acid; AML – amoxicillin; AMP – ampicillin; C – chloramphenicol; CN – gentamicin; DA – clindamycin; E – erythromycin; FOX – cefoxitin; P – penicillin; SXT – sulfamethoxazole/trimethoprim; TE – tetracycline
[2]The resistance rate was calculated as the number of intermediate and resistant isolates divided by the total number of isolates
[3]Applied classes of antimicrobial agents: aminoglycosides, penicillins + b-lactamase inhibitors, lincosamides, macrolides, phenicols, tetracyclines, and folate pathway inhibitors

Table 4. PCR for the presence of genes of antimicrobial resistance in *Staphylococcus* strains isolated from broiler chickens and turkeys

Species	Number of positive samples										
	*tet*K	*tet*L	*tet*M	*tet*O	*erm*A	*erm*B	*aac (6')-Ie aph (2")-Ia*	*msr* A/B	*cfr*	*bla*Z	*mec*A
S. cohnii (33)	13	4	1	-	2	-	1	3	1	26	6
S. epidermidis (17)	8	2	-	1	-	1	6	3	1	15	9
S. hominis (4)	-	1	1	-	-	-	-	1	-	4	2
S. saprophyticus (20)	11	5	2	-	-	-	1	-	1	8	1
S. lentus (10)	2	1	6	-	-	3	-	-	-	6	1
S. xylosus (15)	8	2	1	-	-	2	-	1	-	6	5
S. haemolyticus (4)	1	3	-	-	1	-	-	-	-	3	3
S. sciuri (10)	2	3	2	2	1	-	1	2	-	2	4
S. simulans (5)	1	2	3	-	-	-	-	-	-	1	1
S. chromogenes (9)	-	7	1	2	2	-	2	-	-	3	3
total: 127	46	30	17	5	6	6	11	10	3	74	35

Discussion

In the present study, 237 *Staphylococcus* strains were isolated from samples taken from birds in chicken and turkey flocks. Of these, 35.9% isolates were identified as coagulase-positive *Staphylococcus* species and 64.1% as CNS by MALDI-TOF mass spectrometry. The high number of CNS isolated in this study could be due to the fact that CNS are abundant in the normal skin flora and mucosa of animals and some are free-living in the environment (21). Although normally present, several of these commensal and non-pathogenic staphylococci have been implicated in infections (16). CNS has been isolated from clinically infected chickens with cellulitis, granulomas in the liver and lungs, gangrenous dermatitis, or subcutaneous abscesses. *S. xylosus* and *S. simulans* have been recovered from infected bones and in the course of endocarditis (15, 16, 26). These staphylococcal species can also induce subclinical disease with histopathological lesions in the liver, spleen, and intestines of infected chickens (17, 19). The predominant CNS species found in this study were *S. cohnii* and *S. saprophyticus*, which are included among commonly reported skin microbiota in chickens (24). In studies published by various authors, the percentage of coagulase-negative *Staphylococcus* species isolated from poultry samples varies significantly. For example, in a study by Boamah *et al.* (2), the levels of coagulase-negative strains of staphylococci isolated from samples taken from poultry were as follows: *S. sciuri* 42.97%, *S. lentus* 35.94%, *S. xylosus* 4.30%, *S. haemolyticus* 3.91%, *S. saprophyticus* 1.95%, and *S. cohnii* 0.39%. In a report by Simjee *et al.* (25), 38% of the coagulase negative *Staphylococcus* spp. were *S. sciuri*, while *S. lentus* and *S. xylosus* constituted 21% and 14%, respectively. El-Nagar *et al.* (6) in Egypt found the CNS isolate prevalences from poultry samples to be 34.49% for *S. xylosus*, 17.25% for *S. warneri*, 10.34% for *S. epidermidis*, *S. saprophyticus*, *S. simulans*, and *S. hominis*, and 6.9% for *S. capitis*. Coagulase-negative *Staphylococcus* species such as *S. sciuri*, *S. xylosus* or *S. cohnii* are considered important poultry pathogens, particularly because they carry genes encoding antimicrobial resistance.

The use of antibiotics in poultry production has increased in recent years (24). This extensive use of antibiotics in poultry stimulates bacteria to produce mechanisms of antibiotic resistance, both in the resident bacterial population present in birds as saprophytes and in common pathogens. It is estimated that about 70% of pathogenic microorganisms leading to nosocomial infections are resistant to at least one antimicrobial drug which was previously effective (2). Contaminated poultry products may disseminate resistant pathogenic microorganisms to people as food or *via* direct human-animal contact, and a resulting infection brings high treatment costs (29).

In our study, each of the ten CNS *Staphylococcus* phenotypically exhibited some level of resistance to tetracycline, penicillin G, and ampicillin. All the species were most resistant to tetracycline (59.1%) and penicillin G (54.3%). Moreover, 39 (30.7%) of the CNS isolates exhibited simultaneous resistance to three or more classes of antimicrobial agents as Table 3 shows, which is referred to as multi-drug resistance (MDR) (13). It is noteworthy that 15.7% of the tested CNS strains showed resistance to four or even five of the classes of antimicrobial agents used. Methicillin resistance in this study was confirmed by detection of the *mec*A gene, because according to CLSI, oxacillin MIC criteria may have limit values for some CNS, so testing for *mec*A or PBP2a is recommended for some strains (4). The results of this test, shown in Table 4, indicate that the presence of the *mec*A gene was confirmed in 35 of 127 CNS isolates, which is 27.5% of the strains. Poultry farms and henhouses have been described as sources of antimicrobial resistance, as they are highly contaminated by dust containing endotoxins and aerosolised bacteria, including *Staphylococcus* (9). However, the reason methicillin-resistant staphylococci are found among chickens as yet remains unclear.

Methicillin-resistant strains of *Staphylococcus* are still a growing therapeutic problem. The presence of the regulon *mec* promotes the reorganisation of bacterial genomes and to a large extent accelerates the evolution of genomic regions responsible for resistance to antibiotics and disinfectants (8). Methicillin-resistant *S. aureus* (MRSA) strains have a wide variety of resistance genes located in the chromosome as well as in plasmids and transposons (6). Literature data indicate that most strains harbouring the *mec*A gene also contained the *tet* and *aac (6')- Ie-aph (2")* genes, which might be located on the same or an associated genetic element (8). The mechanisms responsible for antimicrobial resistance in CNS are identical to those occurring in *S. aureus* (9). Our study also indicates that all CNS strains in which the *aac (6')- Ie-aph (2")* gene was detected additionally possessed the *mec*A gene and *tet*K or *tet*L genes; the two genes which, in the current study, mediate the most common tetracycline resistance mechanism. The *tet* genes are contained within conjugative transposons that can be transferred horizontally and expressed in Gram-positive and Gram-negative bacteria (14).

Resistance to erythromycin in staphylococci is usually associated with resistance to other macrolides. The genes *erm*A and *erm*B encoding methyltransferases responsible for resistance to macrolides, lincosamides, and type B streptogramins (MLSB phenotype) through modification of the ribosomal target site have been found in staphylococci (22). The *msr*A/B gene displays another mechanism of inducible resistance to erythromycin by encoding an ATP-dependent efflux pump (23). In our study, the presence of the *erm*A gene was detected in six strains belonging to three species (2/33 *S. cohnii*, 1/4

S. haemolyticus, 1/10 *S. sciuri*, and 2/9 *S. chromogenes*). The *erm*B gene was shown in six strains belonging to three *Staphylococcus* species (1/17 *S. epidermidis*, 3/10 *S. lentus*, and 2/15 *S. xylosus*). In addition, the presence of the msrA/B gene was detected in 10 out of 127 CNS strains (3/33 *S. cohnii*, 3/17 *S. epidermidis*, 1/4 *S. hominis*, 1/15 *S. xylosus*, and 2/10 *S. sciuri*). Literature data indicate that the frequent use of macrolide and lincosamide antibiotics, especially in human medicine, have caused a significant increase in resistance to this group of drugs among various bacterial species around the world (31). In our study, phenotypic assessment of the sensitivity of CNS strains to erythromycin and clindamycin showed a relatively small percentage of resistant strains, 14.1% and 5.5%, respectively. This may be due to the fact that these two antibiotics are less commonly used in the treatment of bacterial infections in poultry.

Aminoglycosides are one of the classes of antibiotics that are key in the treatment of staphylococcal infections. Inactivation of aminoglycoside antibiotics by aminoglycoside modifying enzymes (AME), such as aminoglycoside phosphotransferase (APH), acetyl-transferases (AAC), and nucleotidyl-transferase (ANT), is the most common mechanism of aminoglycoside resistance (10). The most common AME-encoding gene among *Staphylococcus* species is *aac (6')-Ie-aph (2")*, which can be harboured on a plasmid or chromosome and is often included on transposable elements. The bifunctional enzyme AAC (6')-APH (2") encoded by this gene can inactivate a broad range of aminoglycosides and confers concomitant resistance to gentamycin and most aminoglycosides commonly used in medical practice (12). In our study, the presence of the *aac (6')-Ie-aph (2")* gene was detected in strains belonging to five species (1/33 *S. cohnii*, 6/17 *S. epidermidis*, 1/20 *S. saprophyticus*, 1/10 *S. sciuri*, and 2/9 *S. chromogenes*). The phenotypic resistance rate to gentamycin in our study was 25.2%. This is a relatively high percentage of resistant strains, especially given that gentamycin is currently not used in the treatment of bacterial infections in poultry.

The *cfr* gene encodes resistance to five different classes of antimicrobials: phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (12). It is located on a plasmid, although it can also be embedded in a bacterial chromosome. Coagulase-negative staphylococci are considered to be the most common bacteria to carry *cfr* (12). In a study by Wang *et al.* (30), 20 out of 21 *cfr*-positive CNS isolates from chickens, ducks, and pigs identified in China in 2012 were methicillin-resistant. In our study, the presence of the *cfr* gene was demonstrated in 3 out of 127 CNS strains (1/33 *S. cohnii,* 1/17 *S. epidermidis*, and 1/20 *S. saprophyticus*). The increasing drug resistance of staphylococci in animals can be a real threat to people due to the proven transmission of animal strains to humans. Horizontal gene transfer has been demonstrated between *S. aureus* and *S. intermedius* and between *S. aureus* and CNS staphylococci (8). The transmission of genes encoding resistance to antibiotics is facilitated by their localisation in mobile DNA fragments, and the *cfr* gene is an example of a gene with such a location (11).

Staphylococcal enterotoxins (SE) constitute a family of biologically and structurally related toxins. The ingestion of these toxins results in gastrointestinal effects such as nausea, vomiting, diarrhoea, and abdominal pain. They are produced mainly by coagulase-positive staphylococci. CNS have been reported as a conveying vector for virulence genes and have also been implicated in some cases of food poisoning. In the study by El-Nagar *et al.* (6) on material collected from the poultry production chain, enterotoxin genes (*seb*, *sec* and *see*) were found in five strains (23.8%) of *S. aureus*, with a percentage of 9.5% for *seb* and *sec* and 4.8% for *see*, while *sec* and *see* were found in six (20.6%) CNS isolates, with a percentage of 10.3% for each. The *sec* gene was detected in two isolates of *S. xylosus* and one isolate of *S. simulans*, while *see* was detected in *S. xylosus*, *S. warneri*, and *S. simulans*. In our study, only 3 out of 127 strains tested had genes responsible for the production of one of the five classical staphylococcal enterotoxins. Two *S. hominis* strains contained the gene *see*, responsible for the production of enterotoxin E. The presence of the gene responsible for the production of enterotoxin B *(seb*) was found in one strain of *S. epidermidis*.

Enterotoxin B (SEB) is the toxin most commonly associated with classic food poisoning. Staphylococcal enterotoxin B has been studied as a potential biological warfare agent, because it can be easily aerosolised, is very stable and can cause widespread systemic damage, multi-organ system failure, and even shock and death when inhaled at very high dosages (3). There are also reports of staphylococcal food poisoning in France where enterotoxin E (SEE) was the aetiological agent (20).

In conclusion, the data reported above indicate that coagulase-negative strains of *Staphylococcus* spp. isolated from poultry can be a reservoir of antibiotic resistance genes, in particular the *mec*A gene and determinants of resistance to tetracyclines. The isolates were either phenotypically or genotypically multidrug resistant. CNS are recognised as very important bacteria in food production and preservation, and their presence in food animals is clearly significant for public health due to the possibility of antimicrobial resistant genes in those staphylococci. This appears to be linked to the frequent use of certain groups of antibiotics in particular animal species. Nevertheless, multi-resistant strains can also be isolated from animals that have not been treated with antibiotics.

Testing for the presence of the five classic enterotoxin genes showed that only a small percentage of CNS strains from slaughter poultry had the *seb* and *see* genes. The fact that some strains of the isolates

investigated in the present study can produce enterotoxins may, however, be important for public health.

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