

# Antimicrobial resistance and virulence genes in staphylococci isolated from aviary capercaillies and free-living birds in south-eastern Poland

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## Abstract

**Introduction:** The current study characterises *Staphylococcus* bacteria recovered from dead free-living birds and captive capercaillies kept in south-eastern Poland. The results provide novel information about the antimicrobial resistance phenotype/genotype and the virulence profile of these bacteria. **Material and Methods:** Samples of internal organs were taken from dead birds. *Staphylococcus* strains were identified by matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry. Susceptibility to 13 antibiotics was tested using a standard disc diffusion method on Mueller–Hinton agar. All isolates were screened for the presence of antibiotic resistance genes and staphylococcal enterotoxins (A to E), toxic shock syndrome toxin 1, exfoliative toxins A and B and Panton–Valentine leukocidin. **Results:** A total of 129 bacterial strains belonging to 19 species of the *Staphylococcus* genus were isolated. A relatively high percentage of them resisted fluoroquinolones, tetracyclines, macrolides and  $\beta$ -lactams to a significant degree and harboured the *tetK*, *tetM*, *ermC*, *mphC* and *mecA* genes. Strains of the coagulase-negative *S. sciuri*, *S. xylosus* and *S. cohnii* were isolated with genes encoding enterotoxin A and toxic shock syndrome toxin. **Conclusion:** Both coagulase-positive and coagulase-negative staphylococci isolated from aviary capercaillies and free-living birds have significant pathogenic potential, and greater attention must be paid to the coagulase-negative species, which are still often considered mere contaminants. Virulence factors associated with resistance to antimicrobials, this being multiple in some strains, seem most important because they can be easily transferred between animals, especially those living in a given area.

**Keywords:** *Tetrao urogallus*, free-living birds, methicillin-resistant *Staphylococcus*, antimicrobial resistance genes, enterotoxigenicity, toxic shock syndrome toxin.

## Introduction

Bacteria of the *Staphylococcus* genus are widespread in nature. They are often isolated from water, soil and air. Most species of staphylococci are saprophytes, which are part of the natural microbiota of humans and animals, but they are also important aetiological factors of infections and food poisoning (8). Bacteria of this genus can produce numerous virulence factors, including surface proteins essential for the colonisation process and extracellular toxins responsible for the destruction of tissues and inactivation of host defence mechanisms (2). Coagulase-positive staphylococci are

not very numerous but are quite diverse. The most important species of the genus, in terms of its ability to cause infections in humans and animals, is the coagulase-positive *Staphylococcus aureus* (9, 20). Currently recognised *S. aureus* toxins include 22 enterotoxins, toxic shock syndrome toxin (TSST-1), exfoliatin (A and B) and Panton–Valentine leukocidin (PVL), a pore-forming cytotoxin that targets human and rabbit mononuclear and polymorphonuclear cells. Some of these factors are capable of stimulating nonspecific T-cell proliferation and possess superantigenic activity. They are encoded by plasmids, prophages, pathogenicity islands, genomic islands, or genes located on the

chromosome next to the staphylococcal cassette (SCC) containing methicillin resistance genes (22, 29, 33, 38). A single bacterial strain may produce any of these toxins separately or in various combinations (8). Coagulase-negative staphylococci (CNS) inhabit the gastrointestinal tract and respiratory system and are present as physiological microbiota on the skin and mucous membranes of humans and animals. Their virulence is determined by the bacterial cell structures as well as substances and structures produced extracellularly but integrally connected with the cell, e.g. adhesins (8). Coagulase-negative staphylococci have traditionally been considered commensals; however, many CNS species are now recognised as potential opportunistic pathogens and important reservoirs of antibiotic resistance genes and virulence factors that can be transferred to closely related species such as *S. aureus* (2, 12). *Staphylococcus epidermidis*, referred to as a microorganism “on the verge of pathogenicity and commensalism”, is currently considered one of the most important coagulase-negative species (8). Due to the impact of infections with CNS species in both humans and animals, research interest in them has increased over the past decade (24, 38). All species of birds, both farmed and wild, may suffer from *Staphylococcus* infections. Birds are most often infected through damaged skin, resulting in local inflammatory changes in the skin (dermatitis) and subcutaneous tissue (cellulitis) (1, 21). Infections may also occur *via* the gastrointestinal tract or the aerogenic route. The occurrence and pathogenicity of staphylococci have been widely studied in poultry species (15, 20, 24). While the toxigenic potential of coagulase-negative staphylococci was questioned in older reports, more recent scientific articles provide information on the presence of toxigenic genes in these staphylococcal species (2, 6, 12, 30). Still little is known about the occurrence and pathogenicity of these bacteria in wild bird species, especially endangered species such as the western capercaillie (*Tetrao urogallus* L. 1758, in the Phasianidae family and Galliformes order).

The western capercaillie is a large forest bird found in temperate forests of Europe (including Russia), with a geographic range stretching from the Alps, the Pyrenees, and the Balkans to northern Norway. It has long been a focus of interest of naturalists and hunters. According to the European Union Birds Directive (2009/147/EC), the capercaillie is a species of conservation interest because of the population decline observed in various parts of its distribution. It is considered to be an umbrella species for other mountain birds (37). In Poland it is one of the most important game species in national forests. The population and areas of occurrence of the capercaillie are currently declining because of the destruction and fragmentation of old forests, urbanisation and development of tourism infrastructure, agricultural expansion, predation, hunting pressure, poaching, and water pollution. The perilous

state of the population throughout Poland has led to aviary breeding of capercaillies.

The aim of the study was to determine the frequency of the *Staphylococcus* spp. occurrence in samples collected from dead capercaillies kept in aviaries, and samples taken from other free-living bird species, as well as to conduct a phenotypic and genotypic evaluation of the virulence factors of the isolated strains.

## Material and Methods

**Birds.** The samples were collected from dead free-living birds from the Podkarpackie province and from capercaillies living in the adaptation aviary in the Vistula Forest District near Żywiec and from the Capercaillie Breeding Centre in the Leżajsk Forest District near Krosno, all locations in Poland. Samples were taken from 73 dead capercaillies (adult birds, chicks and embryos). The wild birds belonged to 10 orders and 15 species: Ciconiiformes (white stork, *Ciconia ciconia*, n = 35), Galliformes (common pheasant, *Phasianus colchicus*, n = 46; black grouse, *Lyrurus tetrix*, n = 2), Strigiformes (tawny or brown owl, *Strix aluco*, n = 10), Falconiformes (common kestrel, *Falco tinnunculus*, n = 8), Anseriformes (mute swan, *Cygnus olor*, n = 5), Passeriformes (song thrush, *Turdus philomelos*, n = 5; common blackbird, *Turdus merula*, n = 7; meadow pipit, *Anthus pratensis*, n = 5; and fieldfare, *Turdus pilaris*, n = 4), Piciformes (great spotted woodpecker, *Dendrocopos major*, n = 4), Pelecaniformes (grey heron, *Ardea cinerea*, n = 2), Apodiformes (common swift, *Apus apus*, n = 4), and Accipitriformes (common buzzard, *Buteo buteo*, n = 3; and lesser spotted eagle, *Clanga pomarina*, n = 1). The samples were collected from November 2019 to June 2021. The internal organs of dead birds (heart, liver and spleen), tarsal joints and conjunctival and palatal fissure swabs collected during necropsies and unhatched capercaillie embryos were placed in transport media and transported to the laboratory under refrigerated conditions (Table 1).

**Identification of bacterial strains.** The collected material was plated on a blood agar medium (Blood LAB-AGAR; Biocorp, Warsaw, Poland) and Chapman selective medium (Mannitol Salt LAB-AGAR; Biocorp) 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 of the isolated bacteria was made, involving Gram staining, microscope examination of cell morphology and motility, and determination of the type of haemolysis. Quantitative measurement of the colonies was not performed. The isolated bacteria were stored for further testing at –85°C in 50% (v/v) glycerol in brain heart infusion broth (BHI; Sigma-Aldrich, St. Louis, MO, USA).

**Table 1.** Total number of birds and number and type of organs from which bacteria of the genus *Staphylococcus* were isolated

Bird species	Total examined animals	Number of <i>Staphylococcus</i> strains isolated from individual organs or tissue							
		Heart	Liver	Spleen	Tarsal joint	Conjunctiva	Palatal fissure	Yolk sac	Dead embryo
Capercaillie ( <i>Tetrao urogallus</i> )	73	5	5	9	4	5	7	9	13
White stork ( <i>Ciconia ciconia</i> )	35	3	3	5	3	7	6	-	-
Common pheasant ( <i>Phasianus colchicus</i> )	46	1	3	6	1	5	4	2	-
Tawny owl ( <i>Strix aluco</i> )	10	-	1	2	-	1	2	-	-
Common kestrel ( <i>Falco tinnunculus</i> )	8	1	-	2	-	-	2	-	-
Common blackbird ( <i>Turdus merula</i> )	7	-	-	-	1	1	2	-	-
Mute swan ( <i>Cygnus olor</i> )	5	-	1	-	-	1	1	-	-
Song thrush ( <i>Turdus philomenos</i> )	5	-	-	1	-	-	2	-	-
Grey heron ( <i>Ardea cinerea</i> )	2	-	1	-	-	-	-	-	-
Great spotted woodpecker ( <i>Dendrocopos major</i> )	4	-	-	1	-	-	-	-	-
Common buzzard ( <i>Buteo buteo</i> )	3	-	-	-	-	-	-	-	-
Black grouse ( <i>Lyrurus tetrix</i> )	2	-	-	-	-	-	-	-	-
Common swift ( <i>Apus apus</i> )	4	-	-	-	-	-	-	-	-
Meadow pipit ( <i>Anthus pratensis</i> )	5	-	-	-	-	-	-	-	-
Fieldfare ( <i>Turdus pilaris</i> )	4	-	-	-	-	-	-	-	-
Lesser spotted eagle ( <i>Clanga pomarina</i> )	1	-	-	-	-	-	-	-	-
Total	214	10	14	26	9	20	26	11	13

All *Staphylococcus* strains were identified by matrix-assisted laser desorption/ionization (MALDI)–time-of-flight mass spectrometry using the IVD MALDI Biotyper (Bruker Daltonik, Bremen, Germany), as described by Marek *et al.* (20).

**Susceptibility to antibiotics.** Susceptibility to 13 antibiotics was tested by a standard disc diffusion method on Mueller–Hinton agar plates (CM0337B; Oxoid, Ely, UK) using a bacterial suspension with turbidity adjusted to a 0.5 McFarland standard. The susceptibility of bacteria was determined for the following agents: 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); gentamicin 10 µg (CN10); tetracycline 30 µg (TE30); trimethoprim–sulfamethoxazole 1:19, 25 µg (SXT25); enrofloxacin 5 µg (ENR5); and fusidic acid 5 µg (FD5) (Oxoid). Strains were assigned to susceptible (S), intermediate resistant (I), or resistant (R) categories on the basis of the Guidelines for Susceptibility Testing (37). Resistance rates were calculated as the number of intermediate and resistant isolates divided by the total number of isolates. The analysis of resistance (patterns of resistance) of *Staphylococcus* strains to eight classes of antibiotics ( $\beta$ -lactam, macrolides, aminoglycosides, fluoroquinolones, phenicols, tetracyclines, dihydrofolate

reductase inhibitor and fusidic acid) was performed. For quality control, *S. aureus* ATCC 25923, *S. aureus* ATCC43300 and *S. aureus* ATCC 29213 were used in the disc diffusion tests.

**Bacterial DNA extraction.** Bacteria were stimulated to grow by inoculating them on blood agar with defrosted brain heart infusion broth suspension and glycerol and incubating them at 37°C for 24 h. Single colonies were then inoculated in tryptone soya broth (TSB) medium, (CM0129, Oxoid, Ely, UK), at 37°C for 12 h. Cells from the culture grown overnight in TSB medium were collected by centrifugation at 8,000 rpm at 10°C for 15 min and then the supernatant was discarded. Then the pellet was washed twice with normal saline and centrifuged at 8,000 rpm at 10°C for 15 min. Subsequently, the GeneMatrix Bacterial & Yeast Genomic DNA Purification Kit (EURx, Gdańsk, Poland) protocol was followed.

The obtained bacterial DNA was stained with ethidium bromide, agarose gel electrophoresis was performed, and finally the fluorescence of the preparations was compared with that of a preparation of known concentration.

**Detection of resistance genes.** The PCR primers for the antibiotic resistance genes *mecA*, *mecC*, *bla<sub>Z</sub>*, *ermA*, *ermB*, *ermC*, *tetK*, *tetM*, *tetL*, *tetO*, *msrA/B*, *aac* (6'')/*aph* (2''), *norA*, *cfi*, and *mphC* were designed by Genomed (Warsaw, Poland) and are listed in Table 2.

**Table 2.** Nucleotide sequences and sizes of PCR products of amplified genes

Gene	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	PCR conditions	Reference
<i>mecA</i>	AAAATCGATGGTAAAGGTTGGC AGTTCTGGCACTACCGGATTTGC	533	94°C, 5 min, 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, final extension 72°C for 5 min	(23)
<i>mecC</i>	GAA AAA AAG GCT TAG AAC GCC TC GAA GAT CTT TTC CGT TTT CAG C	138	94°C, 15 min, 30 cycles of 94°C for 30 s, 59°C for 1 min, 72°C for 1 min, final extension for 10 min	(35)
<i>blaZ</i>	ACTTCAACACCTGCTGCTTTC TAGGTTCCAGATTGGCCCTTAG	173	95°C, 3 min, 30 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 30 s, final extension 72°C for 4 min	(7)
<i>ermA</i>	TCT AAAAAG CATGTAAAAGAA TGA TTATAATTATTT GATAGC TTC	645		
<i>ermB</i>	TAACGACGAAACTGGCTAAAA ATCTGTGGTATGGCGGGTAAG	416	95°C, 3 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, final extension 72°C for 5 min	(36)
<i>ermC</i>	AATCGTCAATTCCTGCATGT TAATCGTGGAATACGGGTTTG	299		
<i>tetK</i>	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	360		
<i>tetM</i>	AGTGGAGCGATTACAGAA CATATGTCCTGGCGTGTCTA	158	94°C, 5 min, 30 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, final extension 72°C for 5 min	(18)
<i>tetL</i>	ATAAATTGTTTCGGGTCGGTAAT AACCAGCCAATAATGACAATGAT	1077		
<i>tetO</i>	AACTTAGGCATTCTGGCTCAC TCCCAGTTCATATCGTCA	514		
<i>msrA/B</i>	GCAAATGGTGTAGGTAAGACAAC ATCATGTGATGTAAACAAAAT	399	95°C, 3 min, 35 cycles of 93°C for 30 s, 55°C for 2 min, 74°C for 1 min, final extension 72°C 90 s	(36)
<i>Aac(6')/aph(2'')</i>	CAGAGCCTTGGAAGATGAAG CCTCGTGAATTCATGTTCTGGC	348	94°C, 10 min, 35 cycles of 94°C for 45 s, 60°C for 60 s, 72°C for 60 s, final extension cycle 72°C for 5 min	(39)
<i>norA</i>	TTTGTTTTCAGTGTGAGAATTTATGTTG GGCTTGGTAAAATACAGCTATTAAC	140	94°C, 5 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, final extension 72°C for 5 min	(26)
<i>cfr</i>	TGA AGT ATA AAG CAG GTT GGG AGT CA ACC ATA TAA TTG ACC ACA AGC AGC	746	94°C, 2 min, 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 45 s, final extension 72°C for 1 min	(13)
<i>mphC</i>	GAG ACT ACC AAG AAG ACC TGA CG CAT ACG CCG ATT CTC CTG AT	530	95°C, 5 min, 35 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 2 min, final extension 72°C for 1 min	(16)
<i>sea</i>	ACGATCAATTTTACAGC TGCATGTTTCAGAGTTAATC	544		
<i>seb</i>	GAATGATATTAATTCGCATC TCTTGTGCGTAAGATAAACTTC	416	94°C, 5 min, 35 cycles of 94°C for 2 min, 57°C for 2 min, 72°C for 1 min, final extension 72°C for 7 min	(22)
<i>sec</i>	GACATAAAAGCTAGGAATTT AAATCGGATTAACATTATCCA	257		
<i>sed</i>	TTACTAGTTTGGTAATATCTCCTT CCACCATAACAATTAATGC	334		
<i>see</i>	ATAGATAAAGTTAAAACAAGCAA TAACTTACCGTGGACCC	170		
<i>tst</i>	ACCCCTGTTCCCTTATCATC TTTCAGTATTGTAACGCC	326		
<i>pvl</i>	ATCATTAGGTAATAATGTCTGGACATGATCCA GCATCAASTGTATTGGATAGCAAAAGC	433	95°C, 5 min, 30 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min, final extension at 72°C for 5 min	(38)
<i>eta</i>	GCAGGTGTTGATTTAGCATT AGATGTCCCTATTTTGTCTG	93		
<i>etb</i>	ACAAGCAAAAAGAATACAGCG GTTTTGGCTGCTTCTCTTG	226		

An internal control was integrated into each PCR to verify the efficiency of the amplification and to ensure that there was no significant PCR inhibition.

**Genotypic analysis of virulence.** All isolates were screened for the presence of nine virulence genes by PCR amplification with the primers listed in Table 2 used at 0.04  $\mu\text{mol}$  concentration. The following determinants were tested: staphylococcal enterotoxins A to E (*sea*, *seb*, *sec*, *sed* and *see*), toxic shock syndrome toxin 1 (*tst*), exfoliative toxins A and B (*eta* and *etb*) and Pantón–Valentine leukocidin (*pvl*). The conditions of the multiplex PCR reaction followed those from the studies by Garofalo *et al.* (7), Kehrenberg and Schwarz (13), Luthje and Schwarz (16), Malhotra-Kumar *et al.* (18), Murakami *et al.* (23), Stegger *et al.* (35), Sutcliffe *et al.* (36), Ünal and Çınar (38), and Vakulenko *et al.* (39). For quality control, *S. aureus* ATCC43300; *S. aureus* ATCCBAA-2312; *S. haemolyticus* 955; *E. faecium* PE1,

*S. pyogenes* 7020, *E. faecium* ET18, *E. faecalis* JH2-2TET, *S. aureus* RN4220, *S. aureus* MSSA476, ATCC29213, and CCRI-8926; *S. aureus* FRI913; ATCC13566; *S. aureus* FRI151m; *S. aureus* FRI1169, CCM7056; and *S. aureus* 16575 were used in the PCR reactions.

## Results

**Occurrence of *Staphylococcus* species.** A total of 129 bacterial strains belonging to the genus *Staphylococcus* were isolated from the internal organs of 214 capercaillies and free-living birds. The *Staphylococcus* strains isolated from the samples belonged to 19 species. Coagulase-negative strains accounted for 93%, while the remaining 7% were coagulase-positive species.

**Table 3.** Phenotypic antimicrobial resistance of *Staphylococcus* strains

Species		Antibiotic												
		AML25	AMC30	AMP10	P10	FOX30	DA2	C30	E15	CN10	TE30	SXT25	ENR5	FD5
<i>S. sciuri</i> , n = 56	R	15	8	18	16	14	2	2	-	1	15	3	26	28
	I	-	-	-	-	-	12	-	5	1	1	-	-	-
<i>S. xylosus</i> , n = 14	R	3	2	3	3	1	-	2	4	-	6	2	6	6
	I	-	-	-	-	-	1	1	2	-	-	-	-	-
<i>S. equorum</i> , n = 12	R	1	-	1	1	1	1	2	2	-	3	1	1	2
	I	-	-	-	-	-	-	-	3	1	-	-	-	-
<i>S. saprophyticus</i> , n = 8	R	-	1	2	1	-	-	-	5	-	7	2	5	1
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. aureus</i> , n = 6	R	1	4	4	4	-	-	-	-	-	-	-	3	1
	I	-	-	-	-	-	3	-	4	-	-	-	-	-
<i>S. epidermidis</i> , n = 6	R	1	1	4	3	-	3	-	4	-	4	5	5	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. cohnii</i> , n = 6	R	3	1	3	2	3	2	-	3	-	3	2	3	2
	I	-	-	-	-	-	1	-	2	1	-	-	-	-
<i>S. pseudintermedius</i> , n = 3	R	1	1	1	1	-	1	1	1	-	1	-	1	-
	I	-	-	-	-	-	-	-	1	-	-	-	-	-
<i>S. haemolyticus</i> , n = 3	R	1	-	2	2	1	1	-	1	1	1	2	1	1
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. lentus</i> , n = 3	R	-	-	-	-	-	-	-	-	-	1	-	1	1
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. kloosii</i> , n = 2	R	-	-	1	-	-	-	-	-	-	1	-	-	1
	I	-	-	-	-	-	-	-	1	-	-	-	-	-
<i>S. succinus</i> , n = 2	R	-	-	-	-	-	-	-	1	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. vitulinus</i> , n = 2	R	-	1	1	1	1	-	-	1	-	-	-	1	1
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. condimenti</i> , n = 1	R	1	-	1	1	1	1	1	-	-	-	-	1	1
	I	-	-	-	-	-	-	-	1	1	1	1	-	-
<i>S. nepalensis</i> , n = 1	R	-	-	-	-	-	-	-	-	-	1	-	1	-
	I	-	-	-	-	-	-	1	-	-	-	-	-	-
<i>S. arlettae</i> , n = 1	R	-	-	-	1	-	-	-	1	-	1	-	1	1
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. pasteurii</i> , n = 1	R	-	-	1	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. chromogenes</i> , n = 1	R	1	1	1	1	1	1	-	1	-	1	1	1	1
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. warneri</i> , n = 1	R	-	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-
Total number		28	20	43	37	23	29	9	43	6	47	19	57	47
%		21.7	15.5	33.3	28.7	17.8	22.5	7.0	33.3	4.7	36.4	14.7	44.2	36.4

AML25 – amoxicillin 25  $\mu\text{g}$ ; AMC30 – amoxicillin + clavulanic acid 20 + 10  $\mu\text{g}$ ; AMP10 – ampicillin 10  $\mu\text{g}$ ; P10 – penicillin G 10 units; FOX30 – cefoxitin 30  $\mu\text{g}$ ; DA2 – clindamycin 2  $\mu\text{g}$ ; C30 – chloramphenicol 30  $\mu\text{g}$ ; E15 – erythromycin 15  $\mu\text{g}$ ; CN10 – gentamicin 10  $\mu\text{g}$ ; TE30 – tetracycline 30  $\mu\text{g}$ ; SXT25 – trimethoprim–sulfamethoxazole 1:19, 25  $\mu\text{g}$ ; ENR5 – enrofloxacin 5  $\mu\text{g}$ ; FD5 – fusidic acid 5  $\mu\text{g}$

**Table 4.** Percentage of *Staphylococcus* strains isolated from individual bird species resistant to specific antibacterial agents

Bird species	Number of strains	Antibiotic												
		AML25	AMC30	AMP10	P10	FOX30	DA2	C30	E15	CN10	TE30	SXT25	ENR5	FD5
Capercaillie ( <i>Tetrao urogallus</i> )	57	12 21%	7 12.3%	21 36.8%	18 31.6%	9 15.8%	23 40.4%	4 7%	19 33.3%	4 7%	14 24.6%	11 19.3%	26 45.6%	20 35.1%
White stork ( <i>Ciconia ciconia</i> )	27	5 18.5%	3 11.1%	6 22.2%	5 18.5%	5 18.5%	3 11.1%	2 7.4%	9 33.3%	1 3.7%	12 44.4%	3 11.1%	9 33.3%	12 44.4%
Common pheasant ( <i>Phasianus colchicus</i> )	22	10 45.5%	8 36.4%	12 54.5%	11 50.0%	7 31.8%	3 13.6%	3 13.6%	11 50.5%	-	20 90.9%	5 22.7%	18 81.8%	11 50.5%
Tawny owl ( <i>Strix aluco</i> )	6	-	-	1 16.7%	-	-	-	-	1 16.7%	-	1 16.7%	-	-	1 16.7%
Common kestrel ( <i>Falco tinnunculus</i> )	5	-	-	-	-	-	-	-	1 20.0%	-	-	-	1 20.0%	1 20.0%
Common blackbird ( <i>Turdus merula</i> )	4	-	-	-	-	-	-	-	-	-	-	-	-	-
Mute swan ( <i>Cygnus olor</i> )	3	1 33.3%	1 33.3%	2 66.6%	2 66.6%	2 66.6%	-	-	-	-	-	-	2 66.6%	1 33.3%
Song thrush ( <i>Turdus philomenos</i> )	3	-	1 33.3%	1 33.3%	1 33.3%	-	-	-	1 33.3%	-	-	-	1 33.3%	1 33.3%
Grey heron ( <i>Ardea cinerea</i> )	1	-	-	-	-	-	-	-	1 100%	1 100%	-	-	-	-
Great spotted woodpecker ( <i>Dendrocopus major</i> )	1	-	-	-	-	-	-	-	-	-	-	-	-	-

AML25 – amoxicillin 25 µg; AMC30 – amoxicillin+clavulanic acid 20 + 10 µg; AMP10 – ampicillin 10 µg; P10 – penicillin G 10 units; FOX30 – cefoxitin 30 µg; DA2 – clindamycin 2 µg; C30 – chloramphenicol 30 µg; E15 – erythromycin 15 µg; CN10 – gentamicin 10 µg; TE30 – tetracycline 30 µg; SXT25 – trimethoprim–sulfamethoxazole 1:19, 25 µg; ENR5 – enrofloxacin 5 µg; FD5 – fusidic acid 5 µg. The resistance rate was calculated as the number of intermediate and resistant isolates divided by the total number of isolates

**Table 5.** Multidrug resistance profiles of isolated *Staphylococcus* strains

Species of bird	Number of <i>Staphylococcus</i> strains (% of all isolated strains)	Phenotypic resistance combination pattern (number of classes of antimicrobial agents)	Number of strains
Capercaillie ( <i>Tetrao urogallus</i> )	57 (44.2)	no resistance	10
		β-lactams (1)	6
		macrolides (1)	7
		fusidic acid (1)	6
		fluoroquinolones (1)	1
		fusidic acid, fluoroquinolones (2)	5
		phenicols, tetracyclines (2)	1
		fluoroquinolones, β-lactams (2)	1
		fluoroquinolones, fusidic acid, β-lactams (3)	2
		fluoroquinolones, macrolides, β-lactams (3)	2
		macrolides, β-lactams, tetracyclines (3)	2
		fluoroquinolones, fusidic acid, tetracyclines (3)	1
		fluoroquinolones, fusidic acid, β-lactams, sulfonamide + dihydrofolate reductase inhibitor (4)	1
		fluoroquinolones, tetracyclines, β-lactams, sulfonamide + dihydrofolate reductase inhibitor (4)	1
		fluoroquinolones, fusidic acid, β-lactams, tetracyclines (4)	1
		fluoroquinolones, fusidic acid, β-lactams, macrolides (4)	1
		fluoroquinolones, tetracyclines, macrolides, sulfonamide + dihydrofolate reductase inhibitor (4)	1
		fluoroquinolones, macrolides, β-lactams, sulfonamide + dihydrofolate reductase inhibitor (4)	1
fluoroquinolones, aminoglycosides, β-lactams, sulfonamide + dihydrofolate reductase inhibitor (4)	1		
fluoroquinolones, macrolides, β-lactams, tetracyclines, phenicols (5)	1		
fluoroquinolones, macrolides, β-lactams, tetracyclines, sulfonamide + dihydrofolate reductase inhibitor (5)	2		
fluoroquinolones, macrolides, β-lactams, Fusidic acid, aminoglycosides, sulfonamide + dihydrofolate reductase inhibitor (6)	1		

		fluoroquinolones, macrolides, $\beta$ -lactams, Fusidic acid, aminoglycosides, tetracyclines, phenicols, sulfonamide + dihydrofolate reductase inhibitor (8)	2
		no resistance	7
		$\beta$ -lactams (1)	1
		macrolides (1)	1
		tetracyclines (1)	2
		sulfonamide + dihydrofolate reductase inhibitor (1)	1
		macrolides, tetracyclines (2)	2
		Fusidic acid, tetracyclines (2)	3
		Fusidic acid, fluoroquinolones (2)	1
		macrolides, $\beta$ -lactams (2)	1
White stork ( <i>Ciconia ciconia</i> )	27 (20.9)	fusidic acid, fluoroquinolones, tetracyclines (3)	1
		fusidic acid, fluoroquinolones, $\beta$ -lactams (3)	1
		fusidic acid, fluoroquinolones, $\beta$ -lactams, macrolides (4)	1
		fusidic acid, fluoroquinolones, macrolides, sulfonamide + dihydrofolate reductase inhibitor (4)	1
		fusidic acid, fluoroquinolones, macrolides, $\beta$ -lactams, tetracyclines (5)	1
		fusidic acid, fluoroquinolones, macrolides, tetracyclines, sulfonamide + dihydrofolate reductase inhibitor (5)	1
		fusidic acid, fluoroquinolones, macrolides, $\beta$ -lactams, tetracyclines, phenicols (6)	1
		fusidic acid, fluoroquinolones, aminoglycosides, $\beta$ -lactams, tetracyclines, phenicols (6)	1
		no resistance	1
		phenicols (1)	1
		$\beta$ -lactams, tetracyclines (2)	1
		fluoroquinolones, macrolides, tetracyclines (3)	4
		macrolides, $\beta$ -lactams, tetracyclines (3)	1
		fluoroquinolones, $\beta$ -lactams, tetracyclines (3)	1
		fluoroquinolones, macrolides, fusidic acid, tetracyclines (4)	2
		fluoroquinolones, $\beta$ -lactams, fusidic acid, tetracyclines (4)	4
Common pheasant ( <i>Phasianus colchicus</i> )	22 (17.1)	fluoroquinolones, $\beta$ -lactams, tetracyclines, sulfonamide + dihydrofolate reductase inhibitor (4)	1
		fluoroquinolones, fusidic acid, tetracyclines, sulfonamide + dihydrofolate reductase inhibitor (4)	1
		fluoroquinolones, $\beta$ -lactams, tetracyclines, macrolides (4)	1
		fluoroquinolones, fusidic acid, tetracyclines, macrolides, phenicols (5)	1
		fluoroquinolones, fusidic acid, $\beta$ -lactams, tetracyclines, sulfonamide + dihydrofolate reductase inhibitor (5)	1
		fluoroquinolones, fusidic acid, $\beta$ -lactams, tetracyclines, macrolides, sulfonamide + dihydrofolate reductase inhibitor (6)	1
		fluoroquinolones, fusidic acid, $\beta$ -lactams, tetracyclines, macrolides, phenicols, sulfonamide + dihydrofolate reductase inhibitor (7)	1
		no resistance	4
Tawny owl ( <i>Strix aluco</i> )	6 (4.6)	$\beta$ -lactams (1)	1
		macrolides, fusidic acid, tetracyclines (3)	1
Common kestrel ( <i>Falco tinnunculus</i> )	5 (3.9)	no resistance	4
		fluoroquinolones, fusidic acid, macrolides (3)	1
Common blackbird ( <i>Turdus merula</i> )	4 (3.1)	no resistance	4
		no resistance	1
Mute swan ( <i>Cygnus olor</i> )	3 (2.3)	fluoroquinolones, $\beta$ -lactams (2)	1
		fluoroquinolones, $\beta$ -lactams, fusidic acid (3)	1
Song thrush ( <i>Turdus philomelos</i> )	3 (2.3)	no resistance	1
		fluoroquinolones, fusidic acid (2)	1
		$\beta$ -lactams, macrolides (2)	1
Grey heron ( <i>Ardea cinerea</i> )	1 (0.8)	macrolides, aminoglycosides (2)	1
Great spotted woodpecker ( <i>Dendrocopos major</i> )	1 (0.8)	no resistance	1

The percentages of strains belonging to each species were as follows: *S. sciuri* 43.4%, *S. xylosus* 10.8%, *S. equorum* 9.3%, *S. saprophyticus* 6.2%, *S. aureus* 4.7%, *S. epidermidis* 4.7%, *S. cohnii* 4.7%, *S. lentus* 2.3%, *S. haemolyticus* 2.3%, *S. pseudintermedius* 2.3%, *S. succinus* 1.5%, *S. kloosii* 1.5%, *S. vitulinus* 1.5%, *S. condimenti* 0.8%, *S. nepalensis* 0.8%, *S. arlettae* 0.8%, *S. pasteurii* 0.8%, *S. chromogenes* 0.8%, and *S. warneri* 0.8%. The total numbers of *Staphylococcus* strains (and their

percentages) isolated from the capercaillies and other bird species are presented in Table 4.

**Susceptibility to antibiotics.** Testing of the susceptibility of the isolated strains to 13 selected antimicrobial agents indicated 5 antibiotics and chemotherapeutic agents to which more than 30% of strains showed resistance. These were enrofloxacin (44.2% resistant strains), fusidic acid and tetracycline (36.4% for both), and erythromycin and ampicillin (33.3% for both).

**Table 6.** Presence of genes encoding resistance to antimicrobial agents and staphylococcal toxins in isolated strains

Gene	Staphylococcus species																Total	%				
	<i>S. sci</i> (n=56)	<i>S. xyl</i> (n=14)	<i>S. equ</i> (n=12)	<i>S. sap</i> (n=8)	<i>S. aur</i> (n=6)	<i>S. epi</i> (n=6)	<i>S. coh</i> (n=6)	<i>S. pse</i> (n=3)	<i>S. hae</i> (n=3)	<i>S. len</i> (n=3)	<i>S. klo</i> (n=2)	<i>S. suc</i> (n=2)	<i>S. vit</i> (n=2)	<i>S. con</i> (n=1)	<i>S. nep</i> (n=1)	<i>S. arl</i> (n=1)			<i>S. pas</i> (n=1)	<i>S. chr</i> (n=1)	<i>S. war</i> (n=1)	
<i>mecA</i>	7						1					1								9	7.0	
<i>mecC</i>																						
<i>blaZ</i>	2	3		2	4	2		1									1	1		16	12.4	
<i>msrA/B</i>	3	2	3	3		1	1	2	1							1				17	13.2	
<i>ermA</i>	5											1								6	4.7	
<i>ermB</i>	12	1	2	1			2	2							1					21	16.3	
<i>ermC</i>	7	3	5	1		5	3	1	1		1									27	20.9	
<i>tetK</i>	19	5	4	5	2	4	4	2		2	1				1					49	38.0	
<i>tetM</i>	16	7	5	3	3	1		1		1	1				1	1				40	31.0	
<i>tetL</i>	5							1		1										7	5.4	
<i>tetO</i>	3	1	1					1							1					7	5.4	
<i>cfr</i>		1		1			1			1					1					5	3.9	
<i>norA</i>	6	2	1	1	6	2	1	1												20	15.5	
<i>aac(6')/aph(2'')</i>		3	1				1		1	1										7	5.4	
<i>mphC</i>	7	5		3	2	2	2		1		1									23	17.8	
<i>sea</i>		1			1		1													3	2.3	
<i>seb</i>																						
<i>sec</i>																						
<i>sed</i>																						
<i>see</i>																						
<i>pvl</i>																						
<i>eta</i>																						
<i>etb</i>																						
<i>tst</i>	1																			1	0.8	
																				129		

*S. sci* – *Staphylococcus sciuri*; *S. xyl* – *Staphylococcus xylosus*; *S. equ* – *Staphylococcus equorum*; *S. sap* – *Staphylococcus saprophyticus*; *S. aur* – *Staphylococcus aureus*; *S. epi* – *Staphylococcus epidermidis*; *S. coh* – *Staphylococcus cohnii*; *S. pse* – *Staphylococcus pseudintermedius*; *S. hae* – *Staphylococcus haemolyticus*; *S. len* – *Staphylococcus lentus*; *S. klo* – *Staphylococcus kloosii*; *S. suc* – *Staphylococcus succinus*; *S. vit* – *Staphylococcus vitulinus*; *S. con* – *Staphylococcus condimentii*; *S. nep* – *Staphylococcus nepalensis*; *S. arl* – *Staphylococcus arlettae*; *S. pas* – *Staphylococcus pasteurii*; *S. chr* – *Staphylococcus chromogenes*; *S. war* – *Staphylococcus warneri*

In addition, there were three more antibiotics to which more than 20% of staphylococcal strains showed resistance: penicillin G, clindamycin and amoxicillin. The lowest percentage of resistant strains (less than 10%) was observed in the case of gentamicin and chloramphenicol. Detailed data are presented in Table 3. Analysis of the resistance patterns of isolated strains showed that as many as 14 (10.8%) of them were resistant to five or more of the classes of antimicrobial agents used. These were six strains isolated from capercaillies, four isolated from storks, and four isolated from pheasants. Of the 129 *Staphylococcus* strains tested for antibiotic susceptibility, 33 (25.6%) showed no resistance to any of the 13 antimicrobials. The antimicrobial resistance patterns of the isolates are shown in Table 5.

**Antimicrobial resistance genes.** All isolates were tested for the presence of genes encoding resistance to methicillin, beta-lactamase, tetracycline, macrolide-lincosamide-streptogramin B (MLSB), aminoglycoside and florfenicol/chloramphenicol. The results are presented in Table 6.

Of the 129 *Staphylococcus* strains, 9 had the *mecA* gene: 7/56 *S. sciuri*, 1/6 *S. cohnii* and 1/2 *S. vitulinus*. The presence of the *blaZ* gene determining resistance to  $\beta$ -lactam antibiotics was demonstrated in 16 strains. The *msrA/B* gene encoding an ATP-dependent efflux pump was harboured by 17 strains. The three erythromycin ribosomal methylase genes, *ermA*, *ermB* and *ermC*, were demonstrated in 6, 21 and 27 strains, respectively. The presence of the *mphC* gene was noted in 23 strains. In the case of tetracycline resistance genes, *tetK* was demonstrated in 49 strains and *tetM* in 40 strains, while the *tetL* and *tetO* genes were each found in 7 strains.

Carriage of the aminoglycoside resistance gene *aac(6')/aph(2'')* was observed in 7 strains (belonging to the species *S. xylosus*, *S. equorum*, *S. cohnii*, *S. haemolyticus* and *S. lentus*). The presence of the florfenicol/chloramphenicol resistance gene (*cfr*) and quinolone resistance gene (*norA*) was demonstrated in 5 and 20 strains, respectively.

**Prevalence of toxin genes in *Staphylococcus* isolates.** The results of multiplex PCR for the five classical enterotoxins A–E showed that the genome of three strains (of *S. xylosus*, *S. aureus* and *S. cohnii*) contained the gene *sea*, responsible for the production of enterotoxin A. None of the *Staphylococcus* strains had the genes responsible for the production of enterotoxins B, C, D or E. Similarly, none of the strains had the *eta*, *etb* or *pvl* gene responsible for the production of exfoliatin (A and B) and Pantone–Valentine leukocidin. One strain of *S. sciuri*, isolated from a pheasant, showed the presence of the *tst* gene responsible for the production of toxic shock syndrome toxin (TSST-1).

## Discussion

In the present study, high species diversity ( $n = 19$ ) was detected among staphylococci recovered from capercaillies and free-living bird species, *S. sciuri* (43.4%) predominating followed by *S. xylosus* (10.8%). *Staphylococcus sciuri* constituted more than half of the strains isolated from capercaillies and almost 40% of the strains isolated from storks and pheasants (37% and 36.4%, respectively). Single strains of *S. sciuri* were isolated from the remaining bird species. Of all staphylococci isolated from storks, *Staphylococcus*



*xylosus* strains comprised 22.7%. A small percentage of *S. xylosus* strains was also isolated from capercaillies and pheasants (5.3% and 3.7%, respectively), and single strains of *S. xylosus* were isolated from the remaining bird species (blackbirds, kestrels and owls). *Staphylococcus equorum* was the species of 18.2% of all staphylococci isolated from pheasants and 10.5% of all staphylococci isolated from capercaillies; individual strains of this species were also isolated from storks and swans. Pheasant isolates were *S. saprophyticus* in 27.3% of cases and stork isolates were this species in 7.4% of detections. The remaining coagulase-negative *Staphylococcus* species each accounted for less than 10% of the total isolates (Table 3). Strains of *S. epidermidis* were isolated only from capercaillies and storks, while *S. cohnii* strains were isolated from these birds and pheasants in addition. Only two coagulase-positive species were isolated: *S. aureus*, which constituted 4.6% of all isolates, and *S. pseudintermedius* (2.3%). *Staphylococcus aureus* strains were isolated from three capercaillies, a thrush, a kestrel and a stork, while all three *S. pseudintermedius* isolates were derived from capercaillies. It is not possible to make a direct comparison of the prevalence of staphylococci obtained in this study with other reports, because studies on the prevalence of staphylococci in capercaillies are almost inexistent. Data in the available literature show that *S. sciuri* was also the most frequent CNS species in cloacal or tracheal samples from wild birds in other regions of the world (21, 30). Many authors indicate that this species has a broad host range and is adapted to highly varied habitats (10, 19, 24, 31). The second most frequently recovered species in this study, *S. xylosus*, was especially prevalent among capercaillies, pheasants and blackbirds. This species has also been recovered from birds of prey in Portugal. In that study, *S. saprophyticus*, *S. epidermidis*, *S. haemolyticus* and *S. succinus*, recognised as opportunistic pathogens in human infections, represented 6.2%, 4.6%, 2.3% and 1.5% respectively of the total isolates recovered from wild birds (34).

Knowledge of the antimicrobial phenotype and genotype of staphylococci in natural ecosystems is very important because these bacteria can act as vectors of antimicrobial resistance mechanisms. In the present study, 44.2% of isolates showed resistance to enrofloxacin, which was the highest percentage of resistant strains among all antimicrobials used in the study. A high percentage of quinolone resistance (25% of *S. aureus* and 66.6% of *S. hyicus* strains) was observed by Vidala *et al.* (40) among strains isolated from free-living raptors in Catalonia, Spain. The highest percentage of strains resistant to enrofloxacin in the study described here (81.8%) was isolated from pheasants. In addition, 36.4% of the isolated strains showed resistance to tetracycline and 33.3% to erythromycin; in these cases similarly the highest percentage of resistant strains was found among isolates from pheasants (90.9% and 50%, respectively). In

a study on CNS isolates from wild birds in Spain by Ruiz-Ripa *et al.* (31), the percentage of tetracycline- and erythromycin-resistant strains was less than 10%. Staphylococci isolated from free-living raptors from Catalonia did not show resistance to tetracycline, while all isolated strains of *S. hyicus* showed resistance to doxycycline (40). Beta-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems) are very important drugs because of their effective and broad mode of action against various bacterial pathogens in combination with their low toxicity in humans and animals (25). In our study, the highest percentages of strains resistant to  $\beta$ -lactam antibiotics were demonstrated for ampicillin (33.3%), penicillin G (28.7%), and clindamycin (22.5%). Also, a relatively high percentage of staphylococcal strains were resistant to amoxicillin (21.7%). Similarly, other authors have shown a high percentage of resistance to penicillin G and ampicillin among staphylococcal strains isolated from wild birds and free-living raptors in Spain (31, 40). In the research presented in this article, as in the cases mentioned above, the highest percentage of resistance to  $\beta$ -lactam antibiotics was observed among the strains isolated from pheasants. The high percentage of strains isolated from pheasants showing resistance to certain antibiotics may be related to the fact that some free-living pheasants were reared in aviaries by hunting clubs and then released into the environment. Presumably, birds reared in aviaries undergo prophylactic treatments and receive antibiotic therapy in case of health problems.

Resistance to enrofloxacin and  $\beta$ -lactam antibiotics (penicillin G, cefoxitin and clindamycin) was also observed in two of the three strains isolated from mute swans. Fourteen (10.8%) of the *Staphylococcus* isolates exhibited simultaneous resistance to five or more classes of antimicrobial agents, which is referred to as multi-drug resistance (MDR) (17) (Table 5). Strains resistant to five or more classes of antibiotics were isolated from capercaillies (six strains), storks (four strains) and pheasants (four strains). The results of the antimicrobial susceptibility test of coagulase-negative staphylococci from healthy free-ranging birds in Spain showed that as many as 34% isolates were resistant to at least three different classes of antimicrobial agents (31). This represents an important source of transmission of pathogens and its antimicrobial resistance mechanisms to other species of animals. The occurrence of drug-resistant bacterial strains in free-living birds and aviary capercaillies in our research may be related to the geographical area and the presence of farms, mainly poultry farms. The use of antimicrobials in intensive production is a common practice. In addition, capercaillies kept in aviaries are under the care of a veterinarian, and in the provision of that care antimicrobial agents are used with high frequency, enabling the emergence of MDR bacteria. Another risk factor for drug resistant bacteria dissemination is that other animals, such as rodents, small birds (*e.g.* passerines) or insects that might come into contact

with animal farms or open wastewater drains could be contaminated with MDR pathogens and become both disseminators and sources of infection for wild birds and capercaillies in aviaries.

Some species of staphylococci from animal sources are considered reservoirs of resistance genes. The *mecA* gene, the major determinant of methicillin resistance in staphylococci, is found in both *Staphylococcus aureus* and coagulase-negative staphylococci (11). Molecular detection of *mecA*, typically by polymerase chain reaction (PCR), is regarded as the gold standard for the confirmation of methicillin resistance. Methicillin-resistant strains of *S. aureus* (MRSA) have been isolated from birds, including poultry, domestic pigeons and parrots, and wild birds (1). In our study, the presence of the *mecA* gene was detected in nine (7%) *Staphylococcus* strains, which were isolated from pheasants (three strains), storks (three strains), swans (two strains) and a capercaillie (one strain). The *mecA* gene was only detected in coagulase-negative species (Table 6). Recent studies have shown the existence of a novel *mecA* homologue gene for methicillin resistance (*mecC*), which has been detected in bacteria isolated from humans and farm animals in many European countries, as well as in companion and wild animals. Cattle populations have been recognised as reservoirs for *mecC* strains (9, 10, 28). Interestingly, the *mecC* gene has rarely been reported in bird species. Although in recent years, it has been detected in *S. aureus* isolates from wild chaffinches in Scotland and white storks in Spain (10, 27), the presence of the *mecC* gene was not demonstrated in the present study in any *Staphylococcus* strain isolated from capercaillies or other birds.

The *blaZ* gene was detected in 12.4% (16/129) of isolates and in both coagulase-positive and coagulase-negative kinds. Interestingly, none of the strains analysed showed the presence of both the *blaZ* and *mecA* genes simultaneously. The differences in the resistance percentage between the phenotypic tests of resistance to cefoxitin and the detection of the *mecA* gene (17.8% phenotypic resistance vs 7% *mecA*-positive staphylococci isolates) reflect the heterogeneous expression of  $\beta$ -lactam resistance in *Staphylococcus* isolates from wild bird sources.

The *tetK* and *tetM* genes, with or without *tetL* and *tetO*, generally mediated tetracycline resistance in our study, the phenotype with this resistance being one of the most frequently detected (36.4%). Research on coagulase-negative staphylococci from wild birds in Spain showed that tetracycline resistance was especially high among *S. lentus* isolates (60%) and was mediated by the *tetK*, *tetL* and/or *tetM* genes (31). Tetracycline is one of the most commonly used antibiotic classes in food animals (5). In a study by Larsen *et al.* (14), the prevalence of *tetM* and *tetK* in livestock-associated MRSA in Denmark in 1999–2011 was 99% and 89%, respectively.

Macrolide and lincosamide resistance is mediated by various combinations of *erm*, *msr*, *mphC* and other

genes. The frequency of the *ermA*, *ermB*, and *ermC* genes in *Staphylococcus* strains varies depending on the geographic location and the population from which the strains are isolated. In a population of 493 human macrolide-resistant MRSAs, Schmitz *et al.* (32) found that 82.4% of them contained the *ermA* gene. In contrast, only 4.6% of *Staphylococcus* strains isolated in by the present authors had the erythromycin-resistance gene *ermA*. The *ermC* gene was present in the highest percentage (20.7%) of the analysed strains. In addition, 17.8% of the strains had the *mphC* gene and 16.3% had the *ermB* gene.

The presence of *sea*, *seb*, *sec*, *sed* and *see*, responsible for the production of staphylococcal enterotoxins A, B, C, D and E, respectively, was also analysed in our study. The *sea* and *see* genes are carried in prophages, whereas *sed*, the determinant of toxin D, is of plasmid origin. The *seb* gene, responsible for toxin B production, can be of chromosomal origin or carried by plasmids (22, 33). Our study showed the presence of the gene responsible for the production of enterotoxin A (*sea*) in 3 strains of the *S. cohnii*, *S. xylosus* and *S. aureus* species. These strains were isolated from pheasants, owls and thrushes, respectively. The genes responsible for the production of the remaining enterotoxins (B–E) were not shown in any of the analysed strains. Cunha *et al.* (2) observed that *S. epidermidis* and *S. lugdunensis* were the most toxigenic species among the CNS in their study. Some reports indicate that TSST-1 is also produced by CNS (2, 12). In our study, the presence of the *tst* gene, responsible for the production of toxic shock syndrome toxin, was demonstrated in one strain of *S. sciuri* which was isolated from a capercaillie which had died in the first 24 hours of life. A recent study by Ruiz-Ripa *et al.* (31) on coagulase-negative staphylococci from wild birds in Spain found that two *S. sciuri* isolates recovered from cinereous vultures carried the *tst* gene. *Staphylococcus sciuri* is not considered to be particularly pathogenic to animals, but its danger is apparently increasing. Research indicates that it can cause various infections, such as endocarditis, peritonitis, septic shock, urinary tract infections and wound infections, in both animals and humans (3).

According to Schmitz *et al.* (33), *Staphylococcus* strains that are positive for toxigenic gene detection could be regarded as having potential for toxin production. Besides their noting of toxigenicity in *S. epidermidis* and *S. lugdunensis*, Cunha *et al.* (2) also observed the production of some toxins, i.e. TSST-1, SEB and SEC, by isolated *S. epidermidis* and *S. aureus* strains producing enterotoxins A, B and C, although the presence of these genes was not confirmed by PCR. The PCR technique can detect genes contained in genetic lines, irrespective of their expression (2). Although the gene is present in the microorganism, it may not always be active. Only expression of the mRNA sequence (determined by RT-PCR) which encodes toxin synthesis leaves no doubt as to the microorganism's toxic potential.

Among the staphylococcal species isolated from the aviary capercaillies and free-living birds, the vast majority were coagulase negative. Both coagulase-positive and coagulase-negative staphylococci have significant pathogenic potential. It seems, however, that the most important role is played by virulence factors associated with increasing drug resistance, including raising the incidence of multi-resistant strains.

The relatively high percentage of *Staphylococcus* strains showing a significant degree of resistance to fluoroquinolones, tetracyclines, macrolides and  $\beta$ -lactams, as well as the presence of the *tetK*, *tetM*, *ermC*, *mphC* and *mecA* genes, reveals their importance for public health and zoonotic potential. The potential for the transfer of antibiotic resistant bacteria from wildlife or the environment to humans and domestic animals should not be underestimated. Coagulase-negative staphylococci seem to be natural reservoirs of methicillin-resistant genes, even in environments with very low antimicrobial selection pressure. The isolation of *S. sciuri*, *S. xylosus* and *S. cohnii* strains with genes encoding enterotoxin A and toxic shock syndrome toxin indicates that CNS are also important pathogens with toxigenic potential. This underscores the need to pay greater attention to these microorganisms, which are still often considered mere contaminants.

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