Antibiotic resistance of staphylococci from hares, pheasants and poultry products in East Slovakia and North-East Austria

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Summary

The use of antimicrobial agents contributes to the spread of antimicrobial resistance which becomes a serious threat affecting both human and animal health. In our study, 117 coagulase-positive (43.2%) and 154 coagulase-negative staphylococci (56.8%) isolated from hares (*Lepus europaeus*) hunted in North-East Austria, farm pheasants (*Phasianus colchicus*), mechanically separated poultry (MSP) and deep-frozen poultry (originating in the region of East Slovakia) have been tested for their susceptibility to ten antibiotics by disk diffusion test. The results demonstrate that resistance to at least one antibiotic was found in the majority (72.3%) of staphylococcal isolates tested. The highest number of resistant strains (85.0%) was detected in farm pheasants. In general, staphylococcal isolates were often resistant to penicillin (27.7–52.8%) and ampicillin (19.6–55.6%), although resistance to erythromycin in farm pheasants (48.3%) and resistance to novobiocin in deep-frozen poultry predominated (41.2%). However, methicillin/oxacillin-resistant strains were detected in a much lower frequency (6.5–20.8%). Most strains were simultaneously resistant to 2 antibiotics (29.2% isolates from hares; 31.7% from pheasants; 37.6% from MSP and 15.2% from deep-frozen poultry), but multiresistance was also detected in several strains (7.4%). The most effective antibiotic was vancomycin, to which no staphylococcal isolate was resistant.

Keywords

staphylococci; antibiotic resistance; disk diffusion test

The escalating development of antimicrobial resistance in recent years has led to intensification of discussion about the misuse of antimicrobial agents in human and veterinary medicine, nutrition and agriculture [1–4]. The broad use of antibiotics has created a strong selective pressure, which has consistently resulted in the survival and spread of resistant bacteria worldwide. The emergence of resistance has revealed multiple and complex mechanisms by which resistance genes spread across the bacterial kingdom, with apparent disregard for species barriers. Bacteria have also developed means for stabilizing the resistance phenotype, thus dashing the initial hopes of reversing resistance by simply reducing the use of antibiotics [5].

The possibility of transmission of resistant bacteria or horizontal transfer of genes encoding antimicrobial resistance from animals or plants to humans via food becomes a serious matter of public health concern. Some authors consider poultry, pork, beef and eggs as a main source of antimicrobial resistance for humans [6].

Resistance to commonly used antibiotics is emerging among several bacterial species worldwide. In recent years, there has been much written about emergence of multiresistant MRSA (methicillin-resistant *Staphylococcus aureus*) and MRCNS (methicillin-resistant coagulase-negative staphylococci) [7–9]. Staphylococci are ubiquitous microorganisms widespread in nature and are often isolated from humans and a variety of farm animals, pets, and wild animals, as well as from various food products [10]. *Staphylococcus aureus* is the most important coagulase-positive pathogen from staphylococci due to a combination of toxin-mediated virulence, invasiveness and antibiotic resistance [11]. Coagulase-negative staphylococci (CNS) are mostly normal skin commensals and are much less pathogenic than *S. aureus* [12]. However, they represent a continuously evolving store of resistance genes which can be transferred to *Staphylococcus aureus* [13]. Surveillance of antimicrobial resistance is generally considered to be necessary for providing local data for selection of empirical therapy,

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for assessing the scale of the resistance problem at a local, national or international level, for monitoring changes in resistance rates, for detecting the emergence and spread of new resistances among the human, veterinary, agricultural, nutritional and environmental sectors, and for providing a measure of the effectiveness of any interventions aimed at reducing resistance [14–17].

In this study, the prevalence of resistance to selected antibiotics in coagulase-positive and coagulase-negative staphylococci isolated from samples of hares, farm pheasants, mechanically separated poultry and deep-frozen poultry is reported.

MATERIAL AND METHODS

Bacterial strains and culture media

Staphylococcal strains were isolated by the standard procedure a according to ISO 6888-1 using Baird-Parker agar (Himedia, Mumbai, India) from muscles of abdominal cavity of 13 hares (*Lepus europaeus*) hunted in North-East Austria (in the region Wildendürnbach), from thigh muscles of 14 farm pheasants (*Phasianus colchicus*) bred on a farm in East Slovakia, as well as from 14 samples of mechanically separated poultry (MSP) and from neck skin of 9 samples of deep-frozen poultry produced in a poultry-processing plant in the region of East Slovakia [18]. Based on the colony morphology (grown on Baird-Parker agar for 48 h at 37 °C), 271 staphylococcal strains were selected for the tube coagulase test (Staphylo PK, ImunaPharm, Šarišské Michaľany, Slovakia). Staphylococcal strains were stored in brain heart infusion (BHI; Oxoid, Basingstoke, United Kingdom) – glycerol stock solution (1 : 1) at –20 °C and, before each antibiotic susceptibility testing, they were subcultured on Columbia agar (Oxoid) at 37 °C for 24 h.

Antibiotic susceptibility testing

Disk diffusion test was performed as outlined by the CLSI document M2-A9 [19]. A 0.5 McFarland standard suspension of each isolate was prepared in BHI broth and 0.1 ml of inoculum was spread on the surface of Mueller-Hinton agar (Himedia). Commercially distributed disks (Oxoid) with the following concentrations of antibiotics were added onto inoculated Mueller-Hinton agar plates: penicillin 10 μg (P), ampicillin 10 μg (Amp), methicillin 5 μg (Met), oxacillin 1 μg (Ox), streptomycin 10 μg (S), gentamicin 10 μg (CN), erythromycin 15 μg (E), tetracycline 30 μg (Te), vancomycin 30 μg (Van). Susceptibility to novobiocin 30 μg (NV) was tested in samples of MSP and deep-frozen poultry. Diameters of the zones of inhibition were measured after a 24-h incubation at 37 °C (in the case of oxacillin after 48 h at 35 °C). Susceptibility, intermediate susceptibility or resistance of individual staphylococcal isolates were determined according to the criteria set by CLSI document M100-S16 [20]. The minimum inhibitory concentration (MIC) of vancomycin (Sigma-Aldrich, St. Louis, Missouri, USA) was determined in a strain with a decreased susceptibility to vancomycin by the agar dilution method on Mueller-Hinton agar according to the procedure described by CLSI document M7-A7 [21].

RESULTS AND DISCUSSION

Based upon the evaluation of the tube coagulase test, 117 staphylococcal isolates out of 271 (43.2%) were identified as coagulase-positive (0.0% from hares, 41.7% from farm pheasants, 67.7% from MSP, 63.0% from deep-frozen poultry) and 154 (56.8%) as coagulase-negative staphylococci (100.0% from hares, 58.3% from farm pheasants, 32.3% from MSP, 37.0% from deep-frozen poultry).

A number of 75 (27.7%) of staphylococcal isolates (26.4% from hares, 15.0% from farm pheasants, 30.1% from MSP and 47.8% from deep-frozen poultry) were susceptible to all antibiotics tested. On the other hand, the highest number of staphylococci resistant to at least one antibiotic was detected in farm pheasants (85.0%).

Occurrence of intermediate susceptibility and resistance to 10 antibiotics in coagulase-positive and coagulase-negative staphylococci isolated from all four groups of samples is shown in Tab. 1. Phenotypes of resistence in staphylococci to two and more antibiotics are summarized in Tab. 2 and the percentage of staphylococcal strains resistant to more than one antibiotic is shown in Fig. 1. Multiresistant bacteria were resistant to at least three classes of antibiotics.

In hares, more than a half of all staphylococcal isolates were resistant to Amp (40 isolates) and P (38 isolates). However, resistance to Ox was detected in about one fifth of staphylococcal isolates (15 isolates) and the number of strains resistant to methicillin was even lower (6 isolates). The portion of 29.2% of strains isolated from hares were simultaneously resistant to 2 antibiotics, multiresistance was observed in 4.2% of strains. Monoresistance was detected in 15.3% of strains and 81.8% of them were resistant to E. Intermediate susceptibility was observed in 31 strains (43.1%), most frequently to E and S (19 and 12 strains). Intermediate susceptibility was most frequently de-

tected in strains simultaneously resistant to 2 or 3 antibiotics (61.3% isolates).

In farm pheasants, all staphylococcal isolates showed most frequently resistance to E (48.3%); P (45.0%) and Amp (41.7%). All staphylococcal isolates were susceptible to CN and Van. The portion of 31.7% of staphylococci isolated from farm pheasants were resistant to two antibiotics at the same time. Resistance to three classes of antibiotics was detected in only 3.3% of strains. Intermediate susceptibility was found in 19 strains (31.7%), most frequently in coagulase-negative strains (68.4%). Most strains were intermediate-susceptible to S and E, but there were also three and two strains intermediate-susceptible to Met and Te. Intermediate susceptibility was always detected in a combination with resistance to at least one antibiotic.

Staphylococcal strains isolated from mechanically separated poultry (MSP) were mostly resistant to P (46.2%) and Amp (41.9%). All isolates were susceptible to Van. A relatively high number of staphylococci (14.0%) were resistant to CN (69.2% coagulase-positive and 30.8% coagulase-negative staphylococcal strains). The portion of 37.6% of staphylococcal isolates from MDPM were resistant to two antibiotics (68.6% coagulase-positive and 31.4% coagulase-negative strains). Intermediate susceptibility was found in 17 strains (18.3%), most frequently in coagulase-positive strains (82.4%). Most strains were intermediate-susceptible to E and S, but there were also four coagulase-positive strains intermediate-susceptible to Met, Te and NV. It is important to say that intermediate-susceptibility was found in strains simultaneously resistant to six and eight antibiotics.

On average, all staphylococcal strains from deep-frozen poultry showed most frequently resistance to NV (41.3%). Resistance to Met, CN and Van was not detected. Coagulase-positive strains were resistant to only half of antibiotics tested and most of them were resistant to P, Amp or NV. Coagulase-negative isolates were most frequently resistant to at least one antibiotic (88.2% strains) and multiresistance was detected in 40.0% of them. Intermediate susceptibility was detected in 19 staphylococcal strains (41.3%) and only two (10.5%) of them were coagulase-negative.

A number of phenotypic methods has been recommended for the detection of methicillin resistance. Accurate detection of this resistance in staphylococci by routine methods is difficult because of their heterogeneous expression of resistance to β-lactams and a variable interaction between the factors affecting the expression of resistance, including the agent tested, medium, NaCl concentration, inoculum, incubation temperature, period of incubation and the reading of endpoints [22, 23]. The PCR methods are now regarded as the gold standard for detection of methicillin resistance encoded by *mecA* gene. However, these are not currently available for most routine diagnostic laboratories, in particular when economic consideration is taken into account. Moreover,

Tab. 1. Occurrence of intermediate susceptibility and resistance to individual antibiotics in coagulase-positive and coagulase-negative staphylococci tested.

Origin	Hares		Pheasants				MSP				Deep-frozen poultry			
Number of strains	–		+		–		+		–		+		–	
	72		25		35		63		30		29		17	
	I [%]	R [%]	I [%]	R [%]	I [%]	R [%]	I [%]	R [%]	I [%]	R [%]	I [%]	R [%]	I [%]	R [%]
penicillin	0	52.8	0	64.0	0	31.4	0	55.6	0	26.7	0	17.2	0	29.4
ampicillin	0	55.6	0	60.0	0	28.6	0	49.2	0	26.7	0	17.2	0	23.5
methicillin	8.3	8.3	4.0	0	5.7	14.3	1.6	6.4	0	0	0	0	0	0
oxacillin	0	20.8	0	4.0	0	22.9	0	9.5	0	0	0	6.9	0	35.3
streptomycin	13.9	2.8	8.0	4.0	20.0	5.7	12.7	7.9	3.3	3.3	51.7	0	0	17.6
gentamicin	0	2.8	0	0	0	0	0	14.3	0	13.3	0	0	0	0
erythromycin	26.4	23.6	16.0	48.0	11.4	48.6	12.7	25.4	10.0	20.0	27.6	3.4	23.5	35.3
tetracycline	0	11.1	0	20.0	5.7	28.6	3.2	11.1	0	10.0	0	0	0	35.3
vancomycin	0	1.4*	0	0	0	0	0	0	0	0	0	0	0	0
novobiocin							1.6	9.5	0	16.7	3.4	17.2	0	82.4

(–) – coagulase-negative isolates, (+) – cogulase-positive isolates, I – intermediate susceptibility, R – resistance.
* – not confirmed by MIC.

methicillin-resistance may also be associated with other mechanisms than mecA. These include β-lactamase hyperproduction, methicillinases, acquisition of structurally modified PBPs, or nutritionally deficient small colony variants [24]. Most of these strains have a phenotypic low-level or borderline resistance and their differentiation from heteroresistant *mecA* positive strains is problematic by routine methods. Their clinical significance is doubtful [23].

Although no single method shows 100% of sensitivity and specificity at the detection of oxacillin resistance among staphylococci, many studies recommend the disk diffusion test for preliminary detection of methicillin resistance [25–27]. Because of the difficulties in detecting cross-resistance to methicillin and oxacillin (penicillinase resistant penicillins), the oxacillin disk is now the recommended choice for detecting methicillin-resistant staphylococci [28] in spite of the fact that oxacil-

Tab. 2. Phenotypes of resistance to two and more antibiotics in staphylococci.

Phenotypes of resistance	Hares	Pheasants		MSP		Deep-frozen poultry	
	–	+	–	+	–	+	–
P-Amp	17	6	3	20	6	3	–
P-Ox	1	–	–	–	–	–	–
P-S	–	1	–	–	–	–	–
P-E	–	–	1	1	–	–	–
Amp-Ox	1	–	–	–	–	–	–
Amp-Te	1	–	–	–	–	–	–
Met-Ox	–	–	1	–	–	–	–
Ox-E	1	–	–	–	–	–	–
S-E	–	–	1	1	–	–	–
S-Te	–	–	–	–	1	–	–
E-Te	–	2	4	2	1	–	–
E-NV	–	–	–	–	3	1	2
Te-NV	–	–	–	–	–	–	1
P-Amp-Ox	5	1	–	–	–	1	–
P-Amp-S	1	–	–	–	–	–	–
P-Amp-CN	1	–	–	1	1	–	–
P-Amp-E	2	6	2	2	–	–	–
P-Amp-Te	2	1	–	–	–	–	–
P-Amp-NV	–	–	–	1	–	–	–
Ox-E-Te	–	–	1	–	–	–	–
Ox-E-NV	–	–	–	–	–	–	1
S-CN-E	–	–	1	–	–	–	–
S-Te-NV	–	–	–	–	–	–	3
P-Amp-Met-Ox	2	–	2	–	–	–	–
P-Amp-Ox-CN	1	–	–	–	–	–	–
P-Amp-Ox-Te	–	–	1	–	–	–	–
P-Amp-Ox-NV	–	–	–	–	–	1	2
P-Amp-S-E	–	–	–	1	–	–	–
P-Amp-E-Te	1	1	–	1	1	–	–
P-Ox-E-NV	–	–	–	1	–	–	1
P-Amp-Met-Ox-E	2	–	–	–	–	–	–
P-Amp-Met-Ox-Te	1	–	2	–	–	–	–
P-Amp-Ox-E-Te	–	–	–	1	–	–	–
P-Amp-Ox-E-NV	–	–	–	–	–	–	1
P-Amp-S-E-Van	1*	–	–	–	–	–	–
P-Amp-Met-Ox-CN-NV	–	–	–	1	–	–	–
P-Amp-Met-Ox-E-Te	1	–	–	–	–	–	–
P-Amp-Ox-E-Te-NV	–	–	–	–	–	–	1
P-Amp-Met-Ox-S-CN-Te-NV	–	–	–	1	–	–	–
P-Amp-Met-Ox-S-CN-E-Te-NV	–	–	–	2	–	–	–

(–) – coagulase-negative isolates, (+) – cogulase-positive isolates. * – not confirmed by MIC.

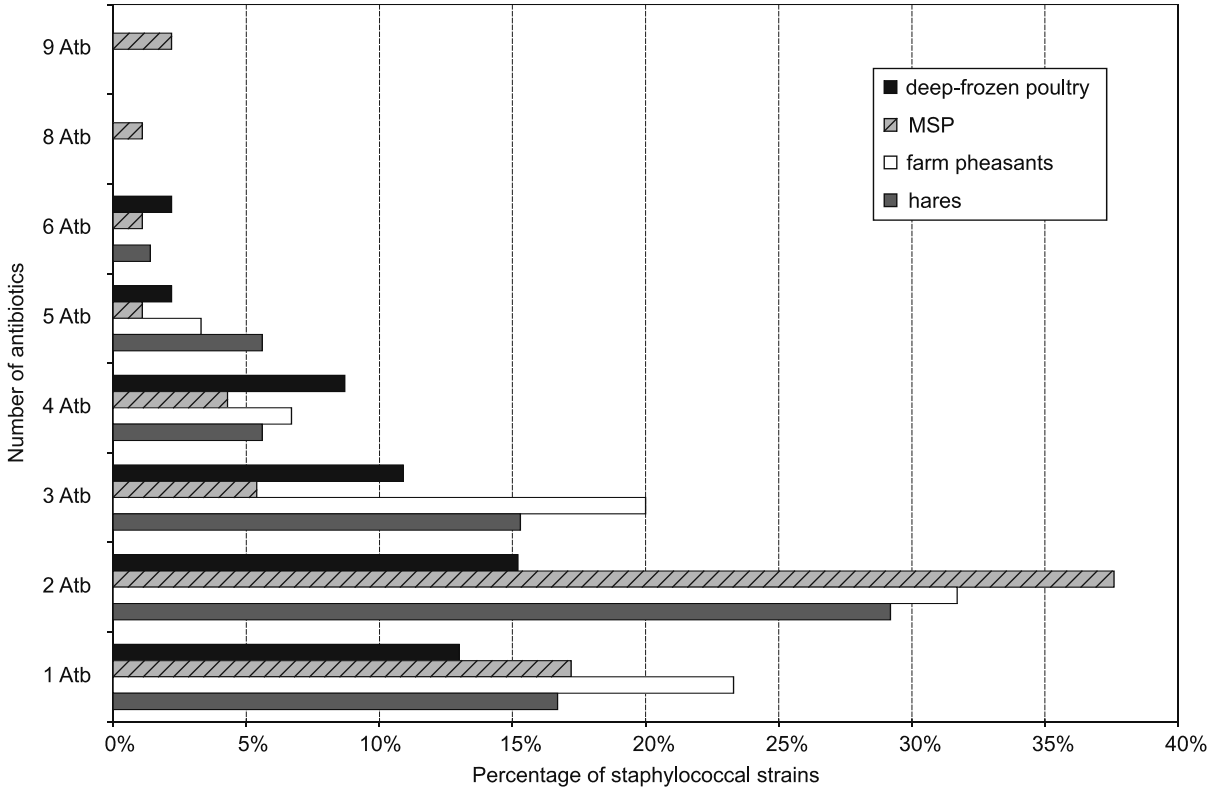

Fig. 1. Occurrence of staphylococcal strains resistant to at least one antibiotic.

lin is less resistant to hydrolysis than methicillin. Determination of methicillin resistance with the help of oxacillin disks was preferred because it is reported that oxacillin maintains its activity longer during storage and it is better available - methicillin is not currently being manufactured in many countries (e.g., USA) [23].

As for occurrence of methicillin/oxacillin resistance in our staphylococcal isolates, 20.8% of oxacillin and 8.3 % of methicillin-resistant strains were isolated from hares, 15.0% of oxacillin and 8.3% of methicillin-resistant strains from farm pheasants, 6.5% of oxacillin and 4.3% of methicillin-resistant strains from MSP, 17.4% of oxacillin and no methicillin-resistant strain from deep-frozen poultry. Resistance to Met was simultaneously detected with resistance to Ox in all cases of methicillin/oxacillin resistance. However, resistance to oxacillin was not always accompanied with resistance to other penicillins (Tab. 2). Our results show that the number of methicillin/oxacillin-resistant staphylococci is more than two times higher when resistance to Ox is taken into account. Most oxacillin-resistant staphylococcal isolates (73.7%) were at the same time resistant to the combination P-Amp. However, few specific profiles of oxacillin resistance have occurred (Tab. 2). The highest number of staphylococcal isolates resistant to P, Amp and Ox were surprisingly detected in hares (16.7%). Similar results were obtained previously in studies on the occurrence of drug-resistant bacteria, in particular commensal bacteria, in wild animals that have never been treated with antibiotics. This finding confirms that resistance to antibiotics has become a global phenomenon and that virtually no region of the earth is unaffected [29, 30].

CONCLUSION

The results demonstrate that the prevalence of antibiotic resistance is relatively high in all commodities tested. Resistance to at least one antibiotic was found in the majority (72.3%) of all staphylococcal isolates tested. The highest portion of resistant strains (85.0%) was detected in farm pheasants. Based upon the results of the disk diffusion test, all staphylococcal strains often showed the resistance to penicillin (27.7–52.8%) and ampicillin (19.6–55.6%). However, methicillin/oxacillin-resistant strains were detected in a much lower frequency (from 6.5% to 20.8%). Surprisingly, the highest number of staphylococcal isolates resistant to P, Amp and Ox were detected

in hares (16.7%). Intermediate susceptibility was detected in all groups of samples tested, most frequently to erythromycin and streptomycin and often in combination with resistance to other antibiotics. Most strains were simultaneously resistant to 2 antibiotics (29.2% isolates from hares; 31.7% from farm pheasants; 37.6% from MSP and 15.2% from deep-frozen poultry), but multiresistance was also detected in several strains (7.4%). The most effective antibiotic was vancomycin, to which no staphylococcal isolate was resistant. Testing the susceptibility of bacteria to antimicrobial drugs is necessary for providing reliable results to the prescribers of antimicrobial drugs and to monitor changes in susceptibility in microbial populations.

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