Antimicrobial resistance of coagulase-negative species of staphylococci isolated from the meat of wild pheasants (Phasianus colchicus)

Ivana Regecová,¹ Monika Pipová,¹ Patricia Jevinová,¹ Vladimir Kmet,² Jana Výrostková,¹ Drahomíra Sopková³
¹Department of Food Hygiene and Technology, University of Veterinary Medicine and Pharmacy, Košice, Slovak Republic
²Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic
³Department of Anatomy, Histology and Physiology, University of Veterinary Medicine and Pharmacy, Košice, Slovak Republic

Abstract

The aim of this study was to determine susceptibility of species-identified coagulase-negative staphylococci (CoNS) isolated from the thigh muscles of hunted wild pheasants (Phasianus colchicus) to seven antibiotics (penicillin, tetracycline, erythromycin, ampicillin, oxacillin, gentamicin, and vancomycin) with the help of agar dilution method. Genus confirmation of each isolate was based on the analysis of PCR product obtained from DNA target 16S ribosomal DNA. Species-identification of staphylococci was performed by means of matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) based on bacterial protein profile. From the results of this study it follows that 41 strains of CoNS were isolated from the meat of wild pheasants. The following species of staphylococci were identified by MALDI-TOF MS: S. epidermidis (17 strains), S. warneri (8 strains), S. haemolyticus (5 strains), S. hominis (4 strains), S. xylosus (3 strains), S. vitulinus (2 strains), S. pasteurii (1 strain) and S. arlettae (1 strain). Based on results of the agar dilution method, resistance to penicillin was detected most frequently (96.2%). On the contrary, 100% susceptibility to vancomycin was observed among isolates of CoNS. Moreover, each out of 41 isolates showed simultaneous resistance to at least two antibiotics tested.

Introduction

In recent years, the food chain has been recognised as a potential hazard in transmission of antibiotic resistant bacteria between animal and human population (Mass, 1986; Ito et al., 2003). Resistant bacteria have been detected in slaughter animals, fish, as well as in various free-living animals. In the case of direct contamination, multiresistant strains occur directly in food-producing animals. However, the food chain can also become contaminated indirectly during food processing, transportation or storage. Thus, both human and animal factors can serve as sources of resistant bacteria (Bardo et al., 2007). Currently, an increasing resistance among strains of Staphylococcus spp. has been reported worldwide. Staphylococci are widespread in the nature. Therefore, they can often be isolated from humans and a variety of farm animals, pets, and wild animals, as well as from various food products (Petráš, 2004). Most strains of pathogenic staphylococci (S. aureus, S. intermedius and S. hyicus) are coagulase-positive (Quinn et al., 1999). Coagulase-negative staphylococci (CoNS) are mostly normal commensals of the skin and mucous membranes (Eff et al., 2002). However, they have been suggested to be a reservoir of antibiotic resistance genes that can be transferred to S. aureus, making it resistant to multiple agents (Perreten et al., 1998). The rate of antimicrobial resistance differs significantly among species of staphylococci. A high number of resistant strains occurs in species which have been identified most frequently as causative agents of nosocomial infections, i.e. S. epidermidis, S. hominis and S. haemolyticus (Droženová and Petráš, 2000).

Currently, no data are available on the species identification of coagulase-negative staphylococci (CoNS) present in the meat of pheasants with the help of matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). So far, little has also been published about resistance of CoNS isolated from the meat of wild pheasants. Therefore, the purpose of this study was to identify species of staphylococci isolated from thigh muscles of wild pheasants (Phasianus colchicus) and to determine the level of their antimicrobial resistance.

Materials and methods

Isolation of staphylococci

Staphylococci were isolated from the thigh muscles of four wild pheasants (Phasianus colchicus) shot during hunting in the Special Facility for Breeding and Diseases of Animals, Fish and Bees in Rozhanovce (managed by the University of Veterinary Medicine and Pharmacy in Košice, East Slovak Republic). Sampling, preparation of test samples, initial suspensions and decimal dilutions for microbiological examination were carried out according to the requirements of STN EN ISO 6881-1 (ISO, 2001b) and STN EN ISO 6882-2 (ISO, 2004). Staphylococci were isolated from the meat of pheasants as described in STN EN ISO 6881-1 (ISO, 2001a) using the Baird-Parker agar medium.

Genus and species identification

The total genomic DNA was isolated from staphylococcal strains as described by Hein et al. (2005) and further tested by polymerase chain reaction (PCR) method according to Strommenger et al. (2003). Primers 16S1 (5-CAGCTCGTGTCGTGAGATGT) and 16S2 (5-AATCATTTGTCCCACCTTTCG) rDNA (Generi Biotech s.r.o., Hradec Králové, Czech Republic) typical for the genus Staphylococcus were used for PCR. The reference strain S. aureus CCM 4223 served as positive control. Reaction mixture in a volume of 50 μL contained 1 μL genomic DNA, 10 mmol/L Tris-HCl (pH 8.8), 3 mmol/L MgCl₂, 200 μmol/L dNTP, 12.5 μmol/L of each primer, and 1 μL Taq DNA polymerase (Ecoli s.r.o., Bratislava, Slovak Republic). The PCR protocol included the following: initial denaturation at 94°C for 3 min, 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min.
When the medium was flooded and acidified producing DNase hydrolysed the deoxyribonuclease was confirmed after a 24-h-incubation at 37°C. Colonies were separated in a 2% agarose gel stained with Goldview TM Nucleic acid stain (Beijing SBS Genetech Co. LTD, Beijing, China) and visualised using the DNR Bio Imaging system (MiniIBIS Pro™; DNR Bio Imaging Systems Ltd., Jerusalem, Israel).

The species of staphylococci were subsequently identified with the help of MALDI BioTyper™ system (Bruker Daltonics, Fremont, CA, USA) based on protein fingerprints measured by MALDI-TOF MS. As staphylococci are Gram-positive bacteria, lysates of bacterial cells had to be prepared according to the instruction of producer (Bruker Daltonics, 2008) prior to their identification.

**Determination of plasma-coagulase and nuclease activity**

Colonies of staphylococci were individually re-inoculated into test tubes containing 2 mL of Brain Heart Infusion Broth (Oxoid, Basingstoke, UK). After 18 to 24 h of incubation at 37°C, 0.1 mL of each bacterial culture was added to another test tube with 1 mL of reconstituted freeze-dried rabbit plasma (Stafylo PK; Imuna Pharm, Šarišské Michaňy, Slovak Republic). Inoculated test tubes were incubated at 37°C. Formation of coagulum was considered as positive reaction. Results were evaluated after 1, 2, 3, 6 and 24 h.

Each isolate was inoculated on the surface of DNase agar (Oxoid) where the production of thermostable deoxyribonuclease was confirmed after a 24-h-incubation at 37°C. Colonies producing DNase hydrolysed the deoxyribonuclease DNA contained in the medium. When the medium was flooded and acidified with 1 N hydrochloric acid, the DNA precipitated, the medium became turbid and clear zones appeared around DNase-positive colonies.

**Results and discussion**

It is generally accepted opinion that increase of bacterial resistance to some antibiotics became explosive and alarming in the last decades. Recently, the food chain has been recognised as one of the main routes for the transfer of antibiotic resistant bacteria between the animal and human population. Antibiotic-resistant strains can be ingested by humans and become a source of genes encoding resistance for bacteria present in the digestive tract (Kožárová et al., 2001; Lee, 2003). Numerous studies already reported on the occurrence of resistant staphylococci in foods and food-producing animals including poultry and game birds.

In this study, 41 strains of staphylococci were isolated from the thigh muscles of wild pheasants, all of them being confirmed as coagulase- and nuclease-negative. The presence of 16S rDNA sequence typical for *Staphylococcus* spp. was determined by PCR method in each isolate. DNA sequence-based species identification is currently the most accurate method for CoNS identification and is considered as the gold standard (Zadoks and Watts, 2009). Even though the 16S rDNA gene is considered to be highly conserved with limited discriminatory power especially in closely related staphylococcal species (Ghebremedhin et al., 2008).

**Table 1. Numbers of resistant, intermediately susceptible and susceptible species of coagulase-negative staphylococci to individual antibiotics tested with details for each species.**

| Species of staphylococci | Penicillin | Oxacillin | Erythromycin | Tetracycline | Ampicillin | Gentamicin | Vancomycin |
|--------------------------|------------|-----------|--------------|--------------|------------|------------|------------|
|                          | R | I | S | R | I | S | R | I | S | R | I | S | R | I | S | R | I | S |
| *S. epidermidis* (n=17)  | 17 | 0 | 0 | 16 | 0 | 1 | 17 | 0 | 0 | 15 | 0 | 2 | 15 | 0 | 2 | 15 | 1 | 0 | 17 |
| *S. warneri* (n=8)      | 7  | 0 | 1 | 6  | 0 | 2 | 8  | 0 | 0 | 5  | 0 | 3 | 3  | 0 | 5 | 8  | 0 | 0 | 8  |
| *S. haemolyticus* (n=5) | 0  | 0 | 0 | 5  | 0 | 0 | 3  | 0 | 0 | 2  | 0 | 0 | 2  | 0 | 3 | 5  | 0 | 0 | 5  |
| *S. hominis* (n=4)      | 2  | 0 | 2 | 3  | 0 | 1 | 3  | 0 | 1 | 2  | 0 | 2 | 1  | 0 | 3 | 3  | 1 | 0 | 4  |
| *S. xylosus* (n=3)      | 3  | 0 | 0 | 3  | 0 | 0 | 2  | 1 | 3 | 0  | 0 | 3  | 0 | 0 | 3  | 0 | 0 | 3  |
| *S. citulinus* (n=2)    | 2  | 0 | 0 | 1  | 0 | 1 | 2  | 0 | 0 | 1  | 0 | 1 | 1  | 0 | 1 | 0  | 1 | 0 | 2  |
| *S. pasteuri* (n=1)     | 1  | 0 | 0 | 1  | 0 | 0 | 1  | 0 | 0 | 1  | 0 | 0 | 1  | 0 | 0 | 1  | 0 | 0 | 1  |

R, resistant; I, intermediately susceptible; S, susceptible.
pheasants. Fifteen of them (79%) were identified as *S. aureus* and *S. intermedius*.

After species-identification, susceptibility to seven antibiotics was tested in all isolates of staphylococci by means of the agar dilution method. As shown in Figure 1, resistance to penicillin was found most frequently in 38 out of 41 isolates of staphylococci from the meat of wild pheasants (92.6%). Frequent resistance to penicillin is also reported in coagulase-positive staphylococci. Lee (2003) found that 28 out of 421 *S. aureus* of major food animals isolates were resistant to oxacillin and 15 of them were positive by PCR for the presence of mecA gene. Three of those isolates came from chickens. According to results of the disk diffusion method, all isolates showed resistance to members of penicillin family, such as penicillin, ampicillin and oxacillin. All isolates of CoNS from the meat of wild pheasants were simultaneously resistant to two or more out of 7 antibiotics tested (Table 1). Sixteen isolates (39.0%) showed simultaneous resistance to 6 antibiotics; vancomycin was the only effective pharmacon. Thirteen isolates of staphylococci (31.7%) were resistant to 5 antibiotics – the most frequent combination (found in 11 strains) was the multiresistance to penicillin, erythromycin, tetracycline, oxacillin, and gentamicin (84.6%). Using the agar dilution method, simultaneous resistance to four, three and two antibiotics was confirmed in 6 (14.6%), 3 (7.3%), and 2 (4.9%) isolates of CoNS, respectively. At present, studies reporting antimicrobial resistance of staphylococci isolated from the meat of pheasants are quite rare. Similarly to our results, Mártonová et al. (2008a) published the results of their study with 138 strains of staphylococci isolated from the meat of hunted pheasants. They confirmed the most frequent resistance to erythromycin (48.3%), penicillin (45.0%) and ampicillin (41.7%). On the other hand, all isolates of staphylococci were sensitive to vancomycin.

Susceptibility to antibiotics differed significantly among species of CoNS tested. All isolates of *S. epidermidis* and *S. haemolyticus* were resistant to penicillin and erythromycin (Table 1). Most of *S. epidermidis* isolates showed resistance to oxacillin (94.1%), followed by resistance to tetracycline, ampicillin, and gentamicin (88.2%). As to *S. warneri* isolates, all of them were resistant to gentamicin and erythromycin. Both isolates of *S. vitulinus* showed simultaneous resistance to all antibiotics tested except for vancomycin. Simultaneous resistance to two and more antibiotics was also confirmed in each of *S. hominis* – one of them showed even resistance to five antibiotics (penicillin-oxacillin-erythromycin-tetracycline-gentamicin). Multiresistance to five antibiotics (penicillin-oxacillin-ampicillin-tetracycline-gentamicin) was also determined in all *S. xylosus* isolates. Isolates of *S. arlettae* and *S. pasteuri* also showed multiresistance to another combination of five antibiotics tested (penicillin, oxacillin, erythromycin, tetracycline, and gentamicin). These results cannot be compared with other published data because they are not yet available.

Conclusions

The results of this study confirm the occurrence of mono- and multiresistant CoNS in the meat of wild pheasants and stress the importance of species identification in CoNS, as the rate of antimicrobial resistance differ significantly among species of staphylococci tested. The study also demonstrates an alarming increase of antimicrobial resistance in staphylococcal isolates from game birds. Therefore, permanent control of the food chain to detect the presence of both causative agents of infections and drug-resistant bacteria, adoption of preventive measures related to environmental hygiene, as well as regular monitoring of actual antimicrobial resistance are effective tools to prevent the spread of antimicrobial resistance worldwide.

References

Bardová, J., Kolář, M., Schlegelová, J., Vágnerová, I., Koukalová, D., Petřízková, J., 2007. Resistence vůči antimikrobálním látkám mezi kmeny Escherichia coli, Staphylococcus spp., Enterococcus spp. izolovaných z potravin živočišného původu. Veterinárníství 4:260-263.

Bruker Daltonics, 2008. MALDI Biotyper 2.0 Software for microorganism identification and classification user manual. Bruker Daltonics Publ., Fremont, CA, USA.

Cain, T., Lubman, D., Weber, J., 1994. Differentiation of bacteria using protein profiles from matrix assisted laser desorption/ionization time of flight mass spectrometry. Rapid Commun. Mass Sp. 8:1026-1030.

CLSI, 2012a. CLSI document M07-A9. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard. 9th rev. ed. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

CLSI, 2012b. CLSI document M100-S22. Performance standards for antimicrobial susceptibility testing. Twenty second informational supplement. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Drozenová, J., Petráš, P., 2000. Vlastnosti koagulázo-negativních stafylokoků izolovaných z hemokultur. Epidemiol. Mikrobiol. Imunol. 49:51-58.

Eiff, C., Peters, G., Heilmann, C., 2002. Pathogenesis of infections due to coagulase-negative staphylococci. Lancet Infect. Dis 2. 11:677-685.

Ghebremedhin, B., Layer, F., König, W., König, B., 2008. Genetic classification and distinguishing of Staphylococcus species based on different partial gap, 16S rRNA, hsp60, rpoB, sodA and tuf gene sequences. J. Clin. Microbiol. 46:1019-1025.
Hájek, V., Balusek, J., Horák, V., Koukalová, D., 1991. Characterization of coagulase-positive staphylococci isolated from free-living birds. J. Hyg. Epid. Microb. Im. 35:407-418.

Hein, I., Jorgensen, H.J., Loncarevic, S., Wagner, M., 2005. Quantification of Staphylococcus aureus in unpasteurised bovine and caprine milk by real-time PCR. Res. Microbiol. 156:554-563.

ISO, 2001a. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species). Part 1: technique using Baird-Parker agar medium. ISO norm 6888-1:1999. International Standardization Organization ed., Geneva, Switzerland.

ISO, 2001b. Microbiology of food and animal feeding stuffs. Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 1: general rules for the preparation of the initial suspension and decimal dilutions. ISO norm 6887-1:1997. International Standardization Organization ed., Geneva, Switzerland.

ISO, 2004. Microbiology of foods and animal feeding stuffs. Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 2: specific rules for the preparation of meat and meat products. ISO norm 6887-2:2003. International Standardization Organization ed., Geneva, Switzerland.

Ito, T., Okuma, K., Ma, X.X., Yuzawa, H., Hiramatsu, K., 2003. Insights on antibiotic resistance of Staphylococcus aureus from its whole genome: genomic island SCC. Drug Resist. Update 6:41-52.

Kožárová, I., Máte, D., Cabadaj, R., 2001. Veterinary drug residues and the safety of foods of animal origin. Folia Vet. 45:214-218.

Lee, J.H., 2003. Methicillin (Oxacillin)-resistant Staphylococcus aureus strain isolated from major food animals and their potential transmission to humans. Appl. Environ. Microb. 69:6489-6494.

Martonová, M., Pipová, M., Jevinová, P., 2008a. Antibiotic resistance of staphylococci from hares, pheasants and poultry products in East Slovakia and North-East Austria. J. Food Nutr. Res. 47:163-169.

Martonová, M., Pipová, M., Laciaková, A., Jevinová, P., 2008b. The use of PCR method in the detection of pathogenicity and antibiotic resistance of staphylococci isolated from farm pheasants. J. Agrobiol. 25:105-107.

Mass, W.K., 1986. Antibiotics in laboratory medicine. 2nd rev. ed. Williams and Wilkins, New York, NY, USA.

Mazzeo, M.F., Sorrentino, A., Gaita, M., Cacace, G., Di Stasio, M., Facchiano, A., Comi, G., Malorni, A., Siciliano, R.A., 2006. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the discrimination of food-borne microorganisms. Appl. Environ. Microb. 72:1180-1189.

Petráš, P., 2004. Actual information in taxonomy of the genus Staphylococcus. Zprávy CEM 13:297-300.

Quinn, P.J., Carter, M.E., Markey, B., Carter, G.R., 1999. Clinical veterinary microbiology. Elsevier, Amsterdam, The Netherlands.

Strommenger, B., Kettlitz, C., Werner, G., Witte, W., 2003. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in Staphylococcus aureus. J. Clin. Microbiol. 41:4089-4094.

Zadoks, R.N., Watts, J.L., 2009. Species identification of coagulase-negative staphylococci: genotyping is superior to phenotyping. Vet. Microbiol. 134:20-28.