

## ANTIMICROBIAL SUSCEPTIBILITY OF OXACILLIN-RESISTANT *STAPHYLOCOCCUS* SPP. ISOLATED FROM POULTRY PRODUCTS

Modestas Ružauskas<sup>1</sup>, Lina Vaškevičiūtė<sup>1</sup>, Rita Šiugždinienė<sup>1</sup>, Irena Klimienė<sup>1</sup>, Marius Virgailis<sup>1</sup>, Rita Plančiūnienė<sup>1</sup>, Lina Anskienė<sup>2</sup>, Raimundas Mockeliūnas<sup>1</sup>, Alvydas Pavilonis<sup>1</sup>

<sup>1</sup> Microbiology and Virology Institute, Lithuanian University of Health Sciences, Tilzes 18, Kaunas, Lithuania, e-mail: ruzauskas@lva.lt

<sup>2</sup> Department of Animal Reproduction, Veterinary Academy, Lithuanian University of Health Sciences, Tilzes 18, Kaunas, Lithuania

### Abstract

The aim of this study was to investigate the prevalence of staphylococci in raw poultry products intended for human consumption and to determine antimicrobial susceptibility, particularly of oxacillin-resistant isolates. Poultry (chicken) liver as representative samples were randomly selected in different retail markets. Isolation of staphylococci was performed using general and selective nutrient media including Mannitol Salt Agar supplemented with cefoxitin as well as Brilliance MRSA2 Agar. Antimicrobial susceptibility testing was carried out using "Sensititre" plates for determination of minimal inhibitory concentrations. Interpretation of results was performed according to CLSI standard. Polymerase chain reaction was used for determination of *mec* genes.

Fifty samples from 70 tested were positive for *Staphylococcus* spp. (71%). In 30% of the isolated staphylococci, the growth was observed on selective media with cefoxitin as well as on MRSA2 Agar. The isolates from those samples were resistant to oxacillin (MIC<sub>50</sub> ≥ 0.5 mg mL<sup>-1</sup>) however, the only one isolate harboured the *mecA* gene. All of the isolates with phenotypic resistance to oxacillin were susceptible to vancomycin, gentamicin, linezolid, daptomicin, rifampin and quinupristin/dalfopristin. The resistances of those isolates were observed to tetracycline (60%), erythromycin (40%), and fluoroquinolones (40%). The species of oxacillin-resistant staphylococci included *S. hyicus* (6), *S. hominis* (4), *S. haemolyticus* (1), *S. cohnii* (1), *S. lentus* (1), *S. warneri* (1) and *S. intermedius* (1). Oxacillin-resistant *Staphylococcus aureus* was not found. The obtained results demonstrated low correlation between phenotypic resistance to oxacillin and the presence of *mec* genes in staphylococcal isolates from the poultry products. Further studies need to be performed for investigation of this unusual finding.

**Keywords:** *Staphylococcus*, *mecA*, resistance, oxacillin, poultry.

### Introduction

*Staphylococcus* spp. are significant bacteria in the etiology of avian diseases and may thus contaminate foods as a result of processed carcasses (Mead, Dodd, 1990; Pepe et al., 2006). Although enterotoxin-producing *S. aureus* is the most common cause of food-borne human illness throughout the world (Do Carmo et al., 2004; LeLoir et al., 2003) the other species such as *S. hyicus*, *S. sciuri*, *S. xylosus* or *S. cohnii* are also important, particularly because of carriage the genes encoding antimicrobial resistance (Aarestrup et al., 2000; Kawano et al., 1996). While staphylococci commonly occur on the skin and nasopharynx of healthy poultry (Mead, Dodd, 1990), they can survive, colonize, and persist at various processing stages in commercial poultry processing plants due to the expression of various key properties, including adhesion (Chaffey, Waites, 1987). Staphylococci are one of the most predominant groups during the slaughtering and processing of poultry, and they have been recovered from air samples (Ellerbroek, 1997), neck skin of chicken carcasses (Geornaras et al., 1995; Olivier et al., 1996), and machinery surfaces (Mead et al., 1995; Huys et al., 2005). By this reason contaminated poultry products could be the source of possible transmission of different staphylococci species including resistant strains to humans, during food processing at home. The aim of this study was to investigate the prevalence of staphylococci in raw poultry products intended for human consumption, to determine species distribution and antimicrobial susceptibility, particularly of oxacillin-resistant isolates.

### Materials and Methods

In 2013 seventy samples of raw poultry liver as representative samples intended for human consumption were randomly selected on different retail markets in Lithuania. The samples were delivered to the laboratory during 1-3 hours. Material was soaked into technically sterile plastic bags containing sterile Tryptone-Soya Broth (Oxoid, Thermo Fisher). Sterile cotton swabs were used for inoculation of outwash onto Sheep Blood Agar, Mannitol Salt Agar (Liofilchem, Italy), Mannitol Salt Agar supplemented with 4 mg L<sup>-1</sup> cefoxitin (Sigma-Aldrich), Brilliance MRSA 2 Agar (Oxoid, Thermo Scientific) as well as on Contrast MRSA Broth (Oxoid, Thermo Scientific). Staphylococci up to the genus level were identified according to morphology characteristics, catalase production, gram-staining, susceptibility to furazolidone. PCR was used for identification of genus specific (16S) subunit. DNA material for molecular testing was obtained after bacterial lysis according to the extraction protocol prepared by the Community Reference Laboratory for Antimicrobial Resistance (Anonymous, 2009) with slight modifications. Briefly, bacterial colonies were taken with a bacteriological loop from the surface of Mueller Hinton Agar and transferred to phosphate buffered saline (pH 7.3). The content was centrifuged for 5 min at 13 000 rpm. Then the supernatant was discarded and the pellet was re-suspended in Tris-EDTA (TE) buffer. The suspension was heated using thermomixer (Biosan) in 100 °C degrees for 10 minutes. Boiled suspension was transferred directly on ice and diluted by 1:10 in TE. Species identification was performed according to pigment and coagulase production, presence of protein

A and clumping factor as well as on biochemical properties using RapID Staph Plus (Thermo Scientific) identification system. In complicated cases species determination was performed by 16S rRNA sequencing using ABI3730XL sequencer. The primers for obtaining of 16S rRNA product (1405 bp) as well as for sequencing were used as follows: forward - 5' GCTCAGGA(CT)GAACGCTGG 3' and the reverse - 5' AGACGATCCWTCAGTGAGC 3' (Couto, 2014). Sequences were analysed using Molecular Evolutionary Genetic Analysis software (MEGA, version 6). Basic local alignment search tool (BLAST) was used for comparison of obtained sequences with sequences presented in the database of National Center of Biotechnology Information.

Antimicrobial susceptibility testing was performed using the broth microdilution method. Sensititre® plates and ARIS 2X automated system (Thermo Scientific) were used with the following antimicrobials: daptomycin, ciprofloxacin, clindamycin, erythromycin, gentamicin, levofloxacin, linezolid, oxacillin, penicillin, co-trimoxazole, quinupristin/dalfopristin and rifampin. Interpretation of results was carried-out using manufacturers software (SWIN®) adapted to clinical breakpoints of Clinical and Laboratory Standards Institute (CLSI). The quality control strain *S. aureus* ATCC 29213 was included in each assay for validation purposes. Detection of *mecA* and *mecA<sub>LGA251</sub>* (*mecC*) genes was performed by PCR described previously (Cuny et al., 2011; Anonymous, 2009).

Statistical analysis was performed using Microsoft Excel software. Comparison between categorical variables was calculated by chi-square and Fisher's exact test. Results were considered statistically significant if  $p < 0.05$ .

**Results and Discussion**

Fifty samples from 70 tested were positive for *Staphylococcus* spp. (71%). In 30% of isolated staphylococci the growth was observed on Mannitol Salt Agar supplemented with cefoxitin as well as on MRSA2 Agar and/or Contrast MRSA Broth. The isolates from those samples were resistant to oxacillin ( $MIC \geq 0.5 \text{ mg L}^{-1}$ ) however the only one isolate harboured the *mecA* gene. All of the isolates phenotypically resistant to oxacillin were susceptible to vancomycin, gentamicin, rifampin, linezolid, daptomycin and quinupristin/dalfopristin. 40% of the isolates demonstrated resistance to at least one fluoroquinolone and erythromycin – the antimicrobials that are treated as critically important for humans. Attention should be paid to this unfavourable finding although it is in coincidence with our previous studies, where high frequency of resistance in other bacterial species (enterococci and *Escherichia coli*) isolated from poultry products has been detected toward fluoroquinolones and/or macrolides (Ružauskas et al., 2010a; Ružauskas et al., 2010b). Resistance to tetracycline was frequent and reached 60%. There are

different data associated with *Staphylococcus* spp. isolated from poultry according to the resistance to tetracycline. For example, Shareef et al. (2009) found that 100% of the isolated *S. aureus* from layers were susceptible to tetracycline, while Jevinova et al., (2009) found that 22% of the *Staphylococcus* spp. from poultry meat demonstrated resistance to this antibiotic. The distributions of minimal inhibitory concentration (MIC) of the oxacillin-resistant isolates are presented in Table 1.

Table 1

**MIC distributions among the isolates (n=15)**

	MIC distributions (%) ( $\text{mg L}^{-1}$ )								
	0.12	0.25	0.5	1	2	4	8	16	32
CIP			67	0	0	33			
CLI	47	20	7	13	7	7			
DAP	7	73	20	7	0				
ERY		0	60	0	0	0	40		
GAT				74	13	13	0	0	
GEN					100	0	0	0	0
LEV		47	20	0	7	13	13	0	
LZD				13	40	47	0	0	
OXA		0	53	33	7	0	7	0	
PEN	40	27	20	13	0	0	0	0	
SYN	0	13	47	20	20	0	0		
RIF			100	0	0	0	0		
TET					33	7	0	60	0
SXT			86	7	7	0	0		
VAN				93	7	0	0	0	0

Grey cells – susceptible; white cells – intermediate; dark cells – resistant

CIP – ciprofloxacin; CLI – clindamycin; DAP – daptomycin; ERY – erythromycin; GAT – gatifloxacin; GEN – gentamicin; LEV – levofloxacin; LZD – linezolid; OXA – oxacillin; PEN – penicillin; SYN – quinupristin/dalfopristin; RIF – rifampin; TET – tetracycline; SXT – co-trimoxazole; VAN – vancomycin.

The species of oxacillin-resistant staphylococci included *S. hyicus* (6), *S. hominis* (4), *S. haemolyticus* (1), *S. cohnii* (1), *S. lentus* (1), *S. warneri* (1) and *S. intermedius* (1). Oxacillin-resistant *Staphylococcus aureus* was not found. Identification of the aforementioned species using biochemical testing was reliable only for 60% of the isolates. For remaining cases sequencing of 16S rRNA was necessary for taxonomic verification.

The obtained results demonstrated low correlation ( $p > 0.05$ ) between phenotypic resistance to oxacillin and the presence of *mecA* gene in staphylococcal isolates from the poultry products. Recently Ba with co-authors (2014) found the similar findings in phenotypically oxacillin-resistant isolates of *Staphylococcus aureus*. Those strains didn't carry neither *mecA* nor *mecC* genes. The authors identified a number of amino acid substitutions present in the

endogenous penicillin binding proteins (PBP) in the resistant strains that were absent in closely related methicillin-susceptible strains. Possibly that substitutions of PBP could be the basis of resistance expression. There are no clear data about similar PBP in other *Staphylococcus* species up to date however, our findings suggest that similar penicillin binding proteins might be expressed in other *Staphylococcus* species as well. Interestingly, in 35 isolates of staphylococci (both coagulase-positive (CPS) and coagulase-negative (CNS) species) isolated from dogs recently (data not presented) we detected *mecA* gene in all of the isolates phenotypically resistant to oxacillin. However, the important aspect for the absence of *mecA* gene in some of the isolates in this study might be associated with different *Staphylococcus* species, where both CNS and CPS are presented. CLSI as well as European Committee on Antimicrobial Susceptibility Testing set oxacillin breakpoint as  $>0.25 \text{ mg L}^{-1}$  only for CNS species, whereas some CPS species had no interpretation criteria. In any case, the results of this study are interesting, therefore further studies need to be performed for the investigation of this unusual finding associated with the absence of *mec* genes in oxacillin-resistant staphylococci prevalent in raw poultry products. Oxacillin-resistant species prevalence in poultry products is interesting as well as some of the species including *S. haemolyticus*, *S. hominis* and *S. cohnii* are prevalent in humans and are well-known because of the high antimicrobial resistance. This fact should be taken into account for safe food production.

### Conclusions

Oxacillin-resistant staphylococci are prevalent in retail poultry products in Lithuanian markets, although oxacillin-resistant *S. aureus* has not yet been detected. The obtained results demonstrated low correlation between phenotypic resistance to oxacillin and the presence of *mec* genes in staphylococcal isolates from the poultry products. All oxacillin-resistant staphylococci were susceptible to vancomycin, gentamicin, linezolid, daptomycin, rifampin, cotrimoxazole and streptogramins. Resistance was observed toward fluoroquinolones (40%), macrolides (40%) and tetracycline (60%). Human-associated *Staphylococcus* species – *S. haemolyticus*, *S. hominis* and *S. cohnii* are prevalent in retail poultry products as well.

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