









Characterization of *Staphylococcus* spp. isolates and β -lactam resistance in broiler chicken production

Caracterização de isolados de *Staphylococcus* spp. e resistência aos β -lactâmicos em isolados oriundos de frango de corte

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Abstract

Methicillin-resistant *Staphylococcus* spp. are important in both human and veterinary medicine due to its resistance to all β -lactam antimicrobials. The *mec* genes (*mecA*, *mecA* variant and *mecC*) and *blaZ* genes are responsible for this phenotype, β -lactam resistance, and are widely spread between staphylococci from animal and human origin. Otherwise, detected that point mutations at the alignment sites could impair the conventional primers for *mecA* gene amplification in some isolates from animal origin. This study aimed to analyze strains of *Staphylococcus* spp. isolated from broiler chicken to evaluate its resistance profile to β -lactams. Sixty cloacal and sixty tracheal swab's were collected at two broiler chicken farms located in the Rio de Janeiro mountain region. The biochemical tests and MALDI-TOF performed bacterial identification. Resistance was evaluated by disk diffusion test and PCR for detecting conventional *mecA*, variant *mecA* and *blaZ* gene. Of 88 staphylococcal isolates, 35,2% (31/88) was identified as *S. gallinarum*, 17% (15/88) of *S. simulans*, 10,2% (9/88) of *S. sciuri*, 4,5% (4/88) of *S. lentus* and *S. cohnii*, and 2,2% of *S. xylosum* and *S. aureus* (only coagulase-positive *Staphylococcus* specie identified). The antimicrobials evaluated were penicillin, cefoxitin, oxacillin and vancomycin. Considering the antibiotype profile of *Staphylococcus* spp. isolates evaluated, six patterns were observed, and the antibiotype 1 were the prevalent presenting 62,5% (55/88). Phenotypic oxacillin-resistance was detected in 26,1% (23/88) of the isolates, and this parameter were used to analyze *mecA* mediated resistance. The conventional primer did not amplify any the *mecA* gene while the universal primer allowed the detection of the variant *mecA* in six strains, being its first report in broilers.

Keywords: antimicrobial resistance, *Staphylococcus* spp., broiler, *mecA*.

Resumo

Staphylococcus spp. resistentes a metilina são importantes na medicina humana e veterinária por serem consequentemente resistentes a todos os antimicrobianos β -lactâmicos. Os genes *mec* (*mecA*, *mecA* variante e *mecC*) e *blaZ* são responsáveis por esse fenótipo, resistência aos β -lactâmicos, e encontram-se difundido nas diferentes espécies de *Staphylococcus* de origem animal e humana. Por outro lado, mutações pontuais nas áreas de anelamento podem impedir os *primers* convencionais de amplificar o gene *mecA* em alguns isolados de origem animal. Este estudo objetivou avaliar o perfil de resistência aos β -lactâmicos em cepas de *Staphylococcus* spp. isoladas de frangos de corte. Um total de 120 amostras, sendo 60 de traqueia e 60 de cloaca foram coletadas em duas granjas de frango de corte localizadas na região serrana do Rio de Janeiro. Provas bioquímicas e MALDI-TOF foram utilizadas na identificação bacteriana. A resistência foi avaliada por teste de difusão em disco e PCR para detecção do *mecA* convencional, *mecA* variante e genes *blaZ*. De 88 cepas isoladas, 35,2% (31/88) foi identificada como *S. gallinarum*. 17% (15/88) de *S. simulans*,




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10.2% (9/88) de *S. sciuri*, 4.5% (4/88) de *S. lentus* e *S. cohnii*, e 2.2% de *S. xylosus* e *S. aureus* (única espécie de *Staphylococcus* coagulase-positiva identificada). Os antimicrobianos avaliados foram penicilina, cefoxitina, oxacilina e vancomicina. Considerando o perfil antibiograma dos isolados de *Staphylococcus* spp. avaliados, seis grupos foram observados, e o antibiograma 1 foi o de maior prevalência apresentando 62.5% (55/88). A resistência fenotípica a oxacilina foi observada em 26,1% (23/88) dos isolados. O *primer* convencional não amplificou qualquer gene *mecA*, enquanto o *primer* universal permitiu a detecção do *mecA* variante em seis cepas, sendo o primeiro relato em frangos de corte.

Palavras-chave: resistência antimicrobiana, *Staphylococcus* spp., frango, *mecA*.

Introduction

Animal production is a relevant activity of Brazilian agribusiness. Brazil stands out in poultry production as the second-largest producer of chicken meat globally, reaching 13.056 million tons, leaving behind only the US with a production of 18.596 million tons (Associação Brasileira de Proteína Animal, 2018). The use of antimicrobials in animal production has different purposes: to protect or improve animal health and to stimulate faster growth and maximize profits (Van Boeckel et al., 2015). The use of antimicrobials as growth promoters is of great concern as it increases the chances of developing resistant bacteria. Many countries have already banned this, especially in the European Union since 2006 (O'Neill, 2015). In Brazil, the prohibition of β -lactams as additives was established in article 18 the Normative Instruction 26/2009 published by Ministry of Agriculture, Livestock and Supplies (Brasil, 2009).

Antimicrobial use is supported due to the importance of infectious diseases causing significant economic losses in poultry production. Both coagulase-positive, especially *S. aureus*, and coagulase-negative *Staphylococcus* spp. (CoNS) has been implicated as an etiological agent in many avian pathologies (Corrand et al., 2012). The clinical signs most associated with *Staphylococcus* spp. in birds are arthritis, synovitis, chondronecrosis and osteomyelitis, gangrenous dermatitis, pododermatitis, omphalitis, septicemia, blepharitis and mandibular osteomyelitis (Huynh et al., 2014).

Methicillin-resistant *Staphylococcus* spp. are important in both human and veterinary medicine once it is related to resistance to all β -lactam antimicrobials (Cohn & Middleton, 2010). Two mechanisms can mediate the resistance to penicillin: the *blaZ* gene that encodes the production of the enzyme β -lactamases, which degrades the β -lactam ring of β -lactam antimicrobials, and the *mecA* gene, that encodes the production of low-affinity penicillin-binding protein called PBP2a or PBP2', whereas this change prevents binding of penicillin, thereby maintaining regular cell wall production (Kuroda et al., 2001). The *mecA* gene is widely spread between both *Staphylococcus* coagulase-positive and negative species from animal and human origin (Huber et al., 2011). Nemeghaire et al. (2014) found a range of 12.5% *Staphylococcus sciuri* methicillin-resistant in broilers reinforcing that CoNS can be reservoirs and help in the dispersion of antimicrobial resistance genes (Schoenfelder et al., 2017).

So, besides the clinical importance of staphylococci antimicrobial resistance, it must be investigated the contribution of poultry production to the worsening of the global antimicrobial resistance framework related to the use of antimicrobials in sub-therapeutic doses and for long periods, establishing ideal conditions for the circulation and maintenance of resistance genes, which can be transmitted to adapted pathogens to the human intestinal microbiota (O'Neill, 2015).

In previous studies developed by the Veterinary Bacteriology Research Group at Federal Rural University of Rio de Janeiro, it was observed that the highly phenotypic resistance to β -lactams was not necessarily correlated to the detection of the *mecA* gene in strains isolated from animals (Mendonça et al., 2012; Soares et al., 2008). Melo et al. (2014) elucidated this puzzling question by detecting a *mecA* gene variant in *Staphylococcus* spp. from bovine in Brazil. From the sequencing and comparison of both variant and classical *mecA* genes, point mutations impair genotypic detection with the previously described primers. The detection of the *mecA* gene variant in strains isolated from different animal species should be expanded to enlarge the understanding of the resistance mediated by such genes in animal origin strains. This study aimed to screen *Staphylococcus* species and its phenogenotypic resistance to β -lactam antimicrobials in isolates from broiler chicken.

Material and methods

Sampling and *Staphylococcus* species identification

A total of 120 samples were collected from two broiler chicken farms in the mountain region of Rio de Janeiro between April 2015 and March 2016. Farms were twice visited in this period and 30 cloacal and 30 tracheal swabs were collected at each time. Swabs were transported in Stuart's medium (Absorve®) at room temperature immediately to LABAC-VET UFRRJ. Samples were inoculated in Blood Agar (HiMedia®) and selective Mannitol Red Phenol Agar (HiMedia®) and incubated at 37 °C for 24h. The isolates were identified in a mass spectrometer (MALDI-TOF LT Microflex Bruker, Bruker) controlled by the FlexControl 3.3 program (Bruker). The spectra were analyzed by the MALDI Biotyper 2.0 (Bruker) program using the standardized configurations for bacterial identification. Scores must be equal or greater than 2 to achieve an acceptable identification. When scores were below 2, isolates were submitted to biochemical identification comprising catalase test, coagulase test, resistance to bacitracin with 0.04 IU disk (SENSIFAR-CEFAR). Coagulase-negative isolates were tested to novobiocin-resistance (5 μ g) (SENSIFAR-CEFAR), and the resistant ones were evaluated by fermentation test of maltose, sucrose, mannitol, mannose, trehalose, xylose, raffinose, and cellobiose (Koneman et al., 2012).

Phenotypic detection of β -lactam resistance

After 18-24 h incubation at 35 °C, colonies were resuspended in saline until a turbidity equivalent to the 0.5 McFarland scale, corresponding to approximately 1.5×10^8 microorganisms/mL. *Staphylococcus aureus* strain ATCC 25923 was used as control (Clinical and Laboratory Standards Institute, 2018). Disk diffusion test were performed according to the methodology recommended by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2018). The antimicrobials tested were penicillin (PEN 10 μ g), cefoxitin (CFO 30 μ g), oxacillin (OXA 1 μ g) and vancomycin (NPV 30 μ g).

DNA extraction

A 1.5 mL aliquot of Brain and Heart infusion Broth with bacterial growth was transferred to microtubes and centrifuged for 5 minutes at 12.000 RPM, and the supernatant was discarded. The procedure was repeated three times. Cells were resuspended in 600 μ l of extraction solution (200 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 1% SDS, 25 mM NaCl) and incubated at 65 °C for 30 min. DNA was extracted with chloroform: isoamyl alcohol 25:24:1 and precipitated with two-volume ice-cold ethanol. The DNA pellet was washed with 70% ethanol and resuspended in 30 μ l of TE buffer (10 mM Tris-HCL, pH 8.0, EDTA 1 mM pH 8.0) and stored at -20 °C until use.

Genotypic detection of β -lactam resistance

The amplification of the classical *mecA* gene was carried out using the primers and methodology described by Murakami et al. (1991): 5'-AAA ATC GAT GGT AAA GGT TGG C-3' and 5'-AGT TCT GCA GTA GG GAT TTG C-3'. The amplification of the *mecA* variant was performed according to Melo et al. (2014): 5'-CAG GCA TGC AGA AAA ATC AA-3' and 5'-TTG AGT CGA ACC AGG TGA TG-3'. A pair of universal primer was used to amplify both classical and variant *mecA* and to analyze its discriminatory power according to Melo et al. (2020): 5'-ACG TTA CAA GAT ATG AAG-3' and 5'-ACA TTA ATA GCC ATC ATC-3'. The amplification of the *bla_Z* gene followed Rosato et al. (2003): 5'-TAC AAC TGT AAT ATC GGA GG-3' and 5'-CAT TAC ACT CTT GGC GGT TT-3'.

Results

A total of 88 *Staphylococcus* spp. strains were isolated from the 120 cloacal and tracheal samples, 36.4% (32/88) from farm 1 and 63.6% (56/88) from farm 2. Isolates were identified by the MALDI

TOF MS, achieving a confident score (2.0 to 2.616), of which 52 isolates needed complementary identification. They were tested for resistance to novobiocin and sugar fermentation, 59.6% (31/52) identified as *S. gallinarum*, and 40.4% (21/52) as other CoNS.

Considering the staphylococci isolate distribution: In farm 1, 37.5% (12/32) of the isolates were from the cloaca and 62.5% (20/32) from the trachea. *S. gallinarum* was the prevalent species comprising 40.6% (13/32), followed by *S. simulans*, *S. lentus*, *S. sciuri*, and other CoNS. In farm 2, 44.6% (25/56) of the isolates were from the cloaca and 55.35% (31/56) from the trachea. *S. gallinarum* was also the prevalent species, presenting 32.1% (18/56) isolates, followed by *S. simulans*, *S. hominis*, *S. cohnii*, *S. lentus*, *S. sciuri*, *S. xylosus*, *S. aureus* and other CoNS that could not be identified at species level (Figure 1).

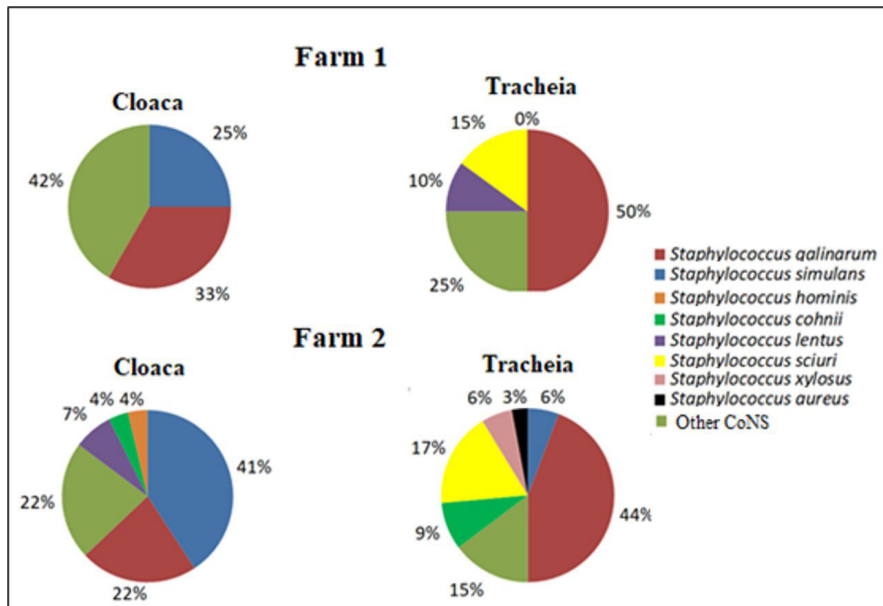


Figure 1. Prevalence of *Staphylococcus* spp. different farms and organs.

Concerning to antimicrobial resistance, a total of 33 (37.5%) *Staphylococcus* isolates (*S. gallinarum*, *S. simulans*, *S. lentus*, *S. sciuri*, *S. xylosus*, *S. cohnii*, *S. aureus* and other CoNS) presented resistance to at least one antimicrobial tested. Of 33, 48.5% (16/33) belongs to the prevalent *S. gallinarum* species. Neither isolates presented resistance to vancomycin. Considering the antibiotype profile of *Staphylococcus* spp. isolates evaluated, six patterns were observed. Antibiotype 1 were the prevalent (62.5%), presenting susceptibility to all antimicrobials tested, followed by antibiotype 2, resistant to oxacillin (13.6%), antibiotype 3, resistant to penicillin (10.2%), antibiotype 4, resistant to penicillin and oxacillin (10.2%), antibiotype 5, resistant to penicillin, cefoxitin and oxacillin (2.3%), and antibiotype 6, resistant cefoxitin (1.2%). Table 1 display the antibiotypes observed at the disk diffusion test.

Table 1. Frequency and β -lactam resistance profile of *Staphylococcus* spp. from broiler chicken.

Antibiotype (n/%)	Resistance profile	Species/number of strains
1 (55/62.5%)	-	<i>S. gallinarum</i> (15)
		Other CoNS (15)
		<i>S. simulans</i> (12)
		<i>S. sciuri</i> (7)
		<i>S. lentus</i> (3)
		<i>S. xylosus</i> (1)
		<i>S. aureus</i> (1)
		<i>S. cohnii</i> (1)

OXA: Oxacillin; PEN: Penicillin; CFO: Cefoxitin; CoNS: Coagulase-negative *Staphylococcus* spp.

Table 1. Continued...

Antibiotype (n/%)	Resistance profile	Species/number of strains
2 (12/13.6%)	OXA	<i>S. gallinarum</i> (5)
		<i>S. simulans</i> (3)
		Other CoNS (2)
		<i>S. lentus</i> (1)
		<i>S. cohnii</i> (1)
3 (9/10.2%)	PEN	<i>S. gallinarum</i> (3)
		<i>S. sciuri</i> (2)
		Other CoNS (2)
		<i>S. aureus</i> (1)
4 (9/10.2%)	PEN, OXA	<i>S. xylosus</i> (1)
		<i>S. gallinarum</i> (7)
		<i>S. cohnii</i> (1)
5 (2/2.3%)	PEN, CFO, OXA	Other CoNS (1)
		<i>S. gallinarum</i> (1)
6 (1/1.2%)	CFO	Other CoNS (1)
		<i>S. cohnii</i> (1)

OXA: Oxacillin; PEN: Penicillin; CFO: Cefoxitin; CoNS: Coagulase-negative *Staphylococcus* spp.

None isolate tested positive for conventional *mecA* gene whereas the variant *mecA* was detected in 6.8% (6/88), of which a singular *S. simulans* from cloaca, and five *S. sciuri* isolates from trachea. One isolate of *S. aureus* (1.1%), from trachea, amplified using universal primer but was not possible to verify the presence of *mec* genes, however, this strain presented the *blaZ* gene. Table 2 displays the results of phenogenotypical β -lactam resistance analysis and the main characteristics of this of *Staphylococcus* isolates.

Table 2. Distribution of phenogenotypical β -lactamic resistance.

Species/n° of strains	Collection sites	Farms	β -lactamic Resistance Detection		
			Phenotypic	Genotypic	
				Types	Universal
<i>S. sciuri</i> (2)	Trachea	1	-	<i>mecA</i> variant	+
<i>S. sciuri</i> (3)	Trachea	2	-	<i>mecA</i> variant	+
<i>S. simulans</i> (1)	Cloaca	2	-	<i>mecA</i> variant	+
<i>S. aureus</i> (1)	Trachea	2	PEN	<i>blaZ</i>	+

PEN: Penicillin; (+): Positive.

Discussion

The most prevalent species identified in both broiler chicken farms was *S. gallinarum* (35.2%, 31/88), followed by *S. simulans* (17.0%, 15/88). Heba et al. (2014), when studying broilers in Egypt, found 5.0% of *S. galinarum* and 22.0% of *S. simulans*. Rueanghiran et al. (2017) found nine different species of CoNS including *S. gallinarum*, but they do not clarify which different species, when analyzed the diversity of *Staphylococcus* spp. in feces of broilers from Thailand.

For a long time, CoNS has been considered harmless and minor important species. However, the advances in molecular taxonomy and systematic allowed a better comprehension of the species dissemination and consequently its pathogenic role (Calazans-Silva et al., 2014). Besides that,

CONS are important reservoir of resistance genes, mainly located on mobile genetic elements, conferring resistance to all classes of antimicrobials which are able to be transfer among different bacterial species and spread further, being a challenge at human and veterinary medicine (Fišarová et al., 2019).

Considering the present study, 23 isolates (26.0%) were methicillin-resistant CoNS at phenotypic analysis, presenting resistance to oxacillin, being 13 (56.5%) *S. gallinarum*. Rueanghiran et al. (2017) detected 52% of methicillin-resistant strains and also considered *S. gallinarum* as a potential source of resistance genes to other bacteria in the environment.

Despite the phenotypic resistance observed, methicillin-resistant expression is peculiar and heterogeneous, and because of this, detection of the *mecA* gene is considered the gold standard method for confirmation of methicillin-resistant isolates by Clinical and Laboratory Standards Institute (2018). However, it was not possible to establish any correlation between phenotypic resistance profiles and the *mecA* gene's presence. Otherwise, universal primer successfully amplified variant *mecA*, showing maximum correlation, confirming that this tool is excellent when screening for *mec*-positives isolates, presenting a high sensitivity and specificity, 93.1% and 98.1% respectively, in a recent study developed by Melo et al. (2020).

The use of universal primer, for conventional and *mecA* variant gene detection, improve methicillin-resistant *Staphylococcus* diagnosis, being crucial for its accurate and rapid identification as proposed by Melo et al. (2020).

Only one isolated presented the *blaZ* gene, that codified the enzyme β -lactamase, showing that the mechanism responsible for β -lactamic resistance, at the farms evaluated, was the expression of *mecA* gene. Besides that, the detection of variant *mecA* gene in broiler chicken samples reinforce the fact that this gene is widespread in the animal environment, mainly in CoNS species.

Despite the phenotypic resistance observed in *S. gallinarum* strains, none of resistance genes (*mecA* genes and *blaZ*) tested in this study was detected in this species. These findings corroborated with Melo et al. (2018), that observed the same pattern evaluating β -lactam resistance in CoNS isolates from bovine intramammary infection. These results could support the hypothesis that these isolates have higher degrees of variability or suffer mutations that prevent its detection requiring further studies to better understanding.

Conclusion

Evaluating the β -lactam resistance in samples from animals requires more specific analyses, including detecting the *mecA* variant, which is not allowed with the conventional *mecA* primers described. This study is the first report of *mecA* variant presence in samples from broiler chicken.

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Ethics statement

The research followed the recommendations established by Ethic Research Committee on non-human vertebrate (CEUA N^o 4401171215).

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Conflict of interests

RLP, DAM, GFB, VRSS, TCNH, SMOC, ISC and MMSS - No conflict of interests.

Authors' contributions

RLP, DAM, GFB, VRSS, TCNH, SMOC, ISC and MMSS- drafted the manuscript. and approved the version to be published. RLP and VRSS - went to the broiler farm collected the samples. RLP, DAM, GFB, VRSS, TCNH- processed the isolates.

Availability of complementary results

All information obtained as a result of the study is included in the manuscript.

The study was carried out at Laboratório de Bacteriologia Veterinária, Departamento de Microbiologia e Imunologia Veterinária, Universidade Federal Rural do Rio de Janeiro.

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