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MULTIPLEX-PCR FOR DETECTION OF β-LACTAM RESISTANCE IN *Staphylococcus spp.*

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ABSTRACT

A Staphylococcus Multiplex PCR system was developed for the simultaneous detection of the *mec*A, *mec*C, *bla*Z (resistance genes of β-lactam resistance) and PVL (pathogenicity factor gene), associated with an internal reaction control with the 16S rRNA gene. There were used primers described in the literature with and without modification and designed primers to standardize the hybridization and amplification temperature of distinct bands with 139 bp (*mec*C), 228 bp (16S), 313 bp (*mec*A), 408 bp (PVL) and 516 bp (*bla*Z) of molecular weight. The standardization was performed in ATCC strains and *Staphylococcus schleiferi* and tested in 56 strains of *Staphylococcus* spp. The 16S gene (internal control) was amplified in all samples, *mec*A gene was detected in two samples, *mec*A associated with *mec*C gene in one sample, *mec*A associated to the *bla*Z gene in 14 samples and the *bla*Z gene in 15 samples. No resistance genes were amplified in 24 samples. The PVL gene was not amplified in any of the samples tested.

KEY-WORDS: genes, *mec*A, *mec*C, *bla*Z, staphylococci

INTRODUCTION

The Polymerase Chain Reaction (PCR) is a technique that allows the in vitro amplification of DNA segments with high speed. It was developed in 1984 by Karry Mullis and was widely used in the diagnosis of infectious and genetic diseases, identification, evolution, and analysis of genetic variability of populations, detection of mutations or polymorphisms, and sequencing of

regions and genomes complete, being inserted in the areas of health, agriculture, biology, biotechnology and archeology (SOLANKI, 2012). The Multiplex PCR is one of the widely used PCR techniques for diagnosis and genotyping. This technique amplifies more than one DNA fragment at the same reaction consisting of the use of multiple primer sets to produce different sizes of amplicons that are specific to different DNA sequences. Because it is a simultaneous amplification of several sequences, the procedure becomes faster, also decreasing operational costs. However, Multiplex PCR requires special care in the design of primers and amplified products. All primers need to work at the same annealing temperature, and the amplified products must have different molecular sizes to be correctly evaluated by electrophoresis.

The bacterias of the genus *Staphylococcus* are important for public health because of their pathogenicity and for being able to acquire mechanisms of resistance easily (LONCARIC et al., 2019; TABATABAEI et al., 2019; HUSE et al., 2018; GUARDABASSI et al., 2013; PATERSON et al., 2014). The main antimicrobial resistance of staphylococci is against the drugs that have the β-lactam ring (penicillins, cephalosporins, monobactams, and carbapenems), and can occur by two mechanisms: the first is the production of a β-lactamase (penicillinase) encoded by the *blaZ* gene, an enzyme that hydrolyzes the peptide bond in the β-lactam ring of the drug, inactivating it. The *blaZ* gene is an integral part of the plasmid, often transferred between bacteria, a phenomenon that facilitates the spread of resistance among staphylococci. This mechanism confers resistance to penicillins (PATERSON et al., 2014).

β-lactam drugs bind irreversibly to Penicillin-Binding Proteins (PBPs) of the membrane, preventing their normal function and leading to the destruction of the bacterial cell wall. The second mechanism of β-lactam resistance in staphylococci is the change on the site of action of β-lactam drugs, mediated by *mec*A or *mec*C (*mec*ALGA251) genes, which encode an altered protein, called Penicillin-Binding Protein 2a (PBP2a) or PBP2'. These enzymes have the function of transpeptidases in the formation of peptidoglycans, which are essential components of the bacterial cell wall. When *mec*A or

*mec*C genes are expressed, the synthesis of PBP2a/PBP2', which has a reduced affinity to all β-lactam drugs, occurs, the binding does not occur, but the enzyme continues to perform the transpeptidation reaction for cell wall biosynthesis, thus ensuring cell integrity in the presence of β-lactam drugs. These genes, *mec*A and *mec*C, are inserted into a genetic element called *Staphylococcal Cassette Chromosome mec* (SCC*mec*) (RUDKIN et al., 2012; PATERSON et al., 2014).

Panton-Valentine leukocidin (PVL) is a phage-borne virulence factor of Staphylococcus (MONECKE et al., 2013). PVL is Leukocidal toxins of two-component, and hetero-oligomeric pore-forming cytolytic toxins (or cytolysin) reveals cytolytic activity with high cell specificity to leukocytes (KANECO and KAMIO, 2004). The presence of PVL is associated with an increase of some isolates that causes leukocyte destruction and tissue necrosis (SZMIEGIELSKI et al., 1999).

The aim of this study was to standardize a Multiplex-PCR for Staphylococcus to detect both β-lactam resistance mediated by *bla*Z, *mec*A and *mec*C genes and to detect the PVL gene, a virulence factor.

MATERIAL AND METHODS

Bacterial

The standardization of temperature and reagent conditions was performed with one *Staphylococcus schleiferi subsp. coagulans* with oxacillin MIC of 64 ug/mL and resistant to penicillin, oxacillin, and cefoxitin in disc diffusion technic. After optimization of the reaction, the PCR was assessed in 9 *Staphylococcus pseudintermedius* known resistant/susceptible by MIC and disc-diffusion and 46 Staphylococcus spp. (including *S. pseudintermedius*, *S. schleiferi*, and Coagulase-positive and -negative *Staphylococcus*, not identified) isolated from clinical animal samples belonging to the library of the Laboratório de Microbiologia Animal (LMA) of Universidade Estadual de Maringá (UEM), Campus Regional de Umuarama (CAU). As control was used American Type Culture Collection (ATCC) of *Staphylococcus aureus* (ATCC 43300) resistant to oxacillin and *Staphylococcus aureus* (ATCC 14458) sensible to oxacillin.

Phenotypic detection of bacterial resistance

The bacterial resistance profile of the studied strains was performed on Muller Hinton Agar (KASVI®) by the disk-diffusion method, according to Bauer et al. (1966). Growth inhibition halos were evaluated according to the norms of the Manual of Clinical and Laboratory Standards Institutes (CLSI) for animal bacteria document VET01S (CLSI, 2018) and also by the Brazilian Committee on Antimicrobial Susceptibility Testing - BrCast (2018) evaluations were carried out for the isolates studied. The antimicrobials tested were: Penicillin G 10U, Oxacillin 1μg, and Cefoxitin 30μg (NEWPROV®).

Determination of Minimum Inhibitory Concentration (MIC)

MIC determination was performed by the Mueller Hinton Broth (DIFCO®) microdilution method, according to CSLI (2015) recommendations using oxacillin (INLAB®). Serial concentrations of 256 to 0.5 μg / mL were used.

DNA extraction

For the bacterial DNA extraction were used 200 μL of bacterial inoculum (107 CFU) incubated with 500 μL of chloroform/isoamyl alcohol (24:1) at 56◦C for 30 min in water bath. After centrifugation at 10,000 × g for 10 min, the supernatant was transferred to another, and a sequence of two baths with 1,000 μL of ice-cold 70° ethanol was realized with homogeneity and centrifugation for 30 seconds at 13,500 x g, where the supernatant was discarded by inversion. Then, the DNA in the microtube was dried in an oven at 56oC for approximately 1 hour. The DNA was eluted in 200μL of ultrapure sterile water and kept at−20◦C until use.

Primers

The multiplex PCR technique was standardized using primers from the literature and designed compatible with multiplex-PCR (Table 1) to detect and differentiate *mec*A, *mec*C, *bla*Z and PVL genes with an internal control by the 16S gene.

Table 1. Primers used for Multiplex PCR to detect β-lactam resistance in *Staphylococcus* spp.

Primer	sequence 5' – 3'	Gene target	product (bp)	Reference
SMAswF (P1)	GATGATACCTTCGTTCCAC	*mec*A	313	SFACIOTTE et al. (2015)
SMAswR (P1)	GTATGTGCGATTGTATTGC			SFACIOTTE et al. (2015)
mecCR	TTA CAC CGA TTC CCA AAT CT	*mec*C	139	Pichon et al. (2012)
mecCFnew	GAT TTA AAG TAG TAG ACG GC			**Drawn**
blaZ-F	AGA GAT TTG CCT ATG CTT C	*blaZ*	516	Modified by ASFOUR and DARWINSH (2011)
blaZ-R	CTT GAC CAC TTT TAT CAG C			Modified by ASFOUR and DARWINSH (2011)
PVLRdraw	ATG TTG CAG TTG TTT TGT AC	PVL	408	**Drawn**
PVLFnew	ACC CCC ATT AGT ACA CAG T			Modified by Pichon et al. (2012)
16SRRNAR	CGC ACA TCA GCG TCA G	16S	228	ASFOUR and DARWINSH (2011)
16SRRNAF	AGG TGG CAA GCG TTA TCC			Modified by ASFOUR and DARWINSH (2011)

Primer designer

The design of the primers was carried out with the software GeneRunner (http://www.generunner.com/).

PCR

Multiplex-PCR reactions were tested with the reagent concentrations shown in Table 2 with 1x PCR Buffer, 10% of the extracted DNA, in a final volume of 25 µl with ultrapure water q.s.p.

Table 2. Concentrations of reagents tested in the standardization of Multiplex-PCR for detection of β-lactam resistance in *Staphylococcus* spp.

Reagents	Concentrations tested			
Primer (pmol for reaction) (Invitrogen®)	0.4	0.2	0.1	0.05
dNTPs (mM of each) (Invitrogen®)	0.4	0.3	0.2	0.2
$MgCl_2$ (mM) (Invitrogen®)	2.0	1.5	1.0	0.5
Platinum Taq DNA polymerase (U) (Invitrogen®)	1.25	1.0	0.5	0.25

Amplification was performed on a thermal cycler (Veriti Applied Biosystems®) at the following time/temperature conditions: an initial denaturation step at 94 ° C for 7 minutes; 40/30 cycles of amplification at 94 ° C for 1 minute, hybridization temperature for 1 minute and 72 ° C for 1 minute; and a final extension step at 72 ° C for 7 minutes, and maintained at 4 ° C until use. Hybridization temperatures, 52/54/56 ° C, were tested in single PCR and multiplex reactions.

Amplified products

Aliquots of 8 µl from the amplified products were submitted to electrophoresis in 2.5% agarose gel in TBE buffer (pH 8.4 – Tris 89 mM, boric acid 89 mM, and EDTA 2 mM) under constant voltage (90 V) for approximately 45 min. The gel was stained with SYBR® Safe DNA gel stain and visualized under UV light.

RESULTS

The results of standardization of hybridization temperature (fig. 1) determined that the ideal temperature of hybridization was 54o C, and the ideal cycling profile was one step of 7 min at 94◦C, followed by 40 cycles of 1 min at 94◦C, 1 min at 54◦C and 1 min at 72◦ C, with one final step at 72◦ C for 7 min. The product was kept at 4◦C.

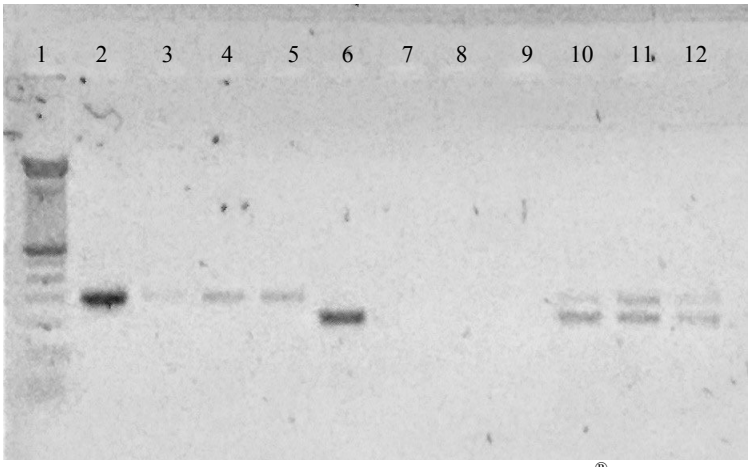

Figure 1. Electrophoresis of agarose gel stained with SYBR® Safe of the PCR Multiplex Standardization. **Line 1**: ladder of 100 bp (Invitrogen®); **Line 2**: *mec*A primers in the ATCC 43300; **Line 3 to 5**: *mec*A primers in the *Staphylococcus schleiferi* - 52º C, 54º C, 56º C; **Line 6**: 16S primers in the ATCC 43300; **Line 7 to 9**: 16S primers in the *Staphylococcus schleiferi* - 52º C, 54º C, 56º C; **Line 10**: *mec*A and 16S primers in the *Staphylococcus schleiferi* - 52º C, 54º C, 56º C; **Line 2 and 6**: 40 cycles of 94º C for 1 min., 54º C for 1 min. and 72º C for 1 min.; **Lines 3 to 5 and 7 to 12**: 30 cycles of 94º C for 30 seg., 52/54/56º C for 30 seg. and 72º C for 30 seg.

The results of standardization of concentrations of (fig. 2) determined that the ideal amplification was obtained using 0.4 pmol of each primer, 0.4 mM of each dNTP, 2 mM of $MgCl_2$, 1x PCR *Buffer,* 1.25 units of *Platinum® Taq* DNA polymerase, 10% of DNA, in a final volume of 25 µL.

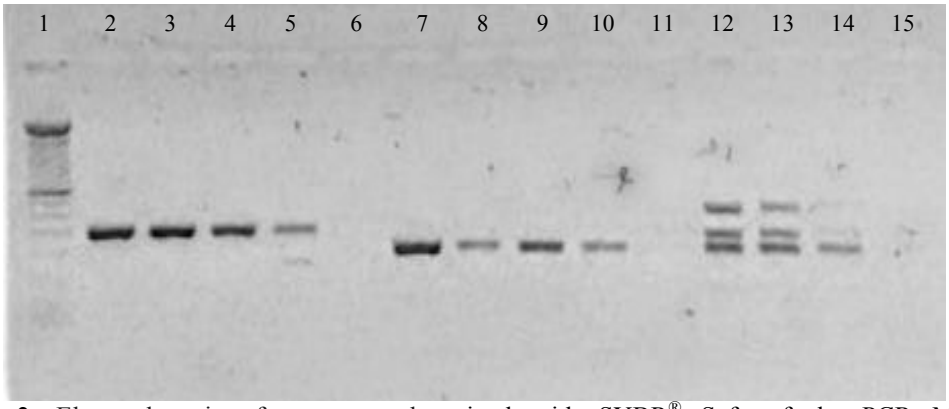

Figure 2. Electrophoresis of agarose gel stained with SYBR® Safe of the PCR Multiplex Standardization. **Line 1**: ladder of 100 bp (Invitrogen®); **Line 2 and 7**: ATCC 43300; **Line 3 to 6 and 8 to 15**: *Staphylococcus schleiferi*; **Line 3 to 6**: *mec*A primers; **Line 7 to 11**: 16S primers; **Line 12 to 15**: *mec*A and 16S primers; **Line 3, 8 and 12**: 0.4 pmol of primer, 0.4 mM of dNTP, 2 Mm of MgCl2 and 1.25 U of polimerase; **Line 4, 9 and 13**: 0,2 pmol of primer; 0,3 mM of dNTP; 1,5 mM of MgCl2; 1U of polimerase; **Line 5, 10 and 14**: 0,1 pmol of primer; 0,2 mM of dNTP; 1 mM MgCl2; 0,5U of polimerase; **Line 6, 11 and 15**: 0,05 pmol de primer; 0,1 mM of dNTP; 0,5 mM of MgCl2; 0,25U of polimerase.

The PCR multiplex successfully detected the resistance/susceptibility to β-lactams drugs in the 9 *Staphylococcus pseudintermedius* isolates from animal infections. The four bacterial strains that presented MIC for oxacillin above 8 ug/mL and were resistant to penicillin, oxacillin, and cefoxitin in disc-diffusion the *mec*A gene was amplified. In three of these samples, the *blaZ* gene was also amplified. The *mec*A gene was not amplified in any of the 5 *Staphylococcus pseudintermedius* with MIC ≤ 0.5 ug/mL (Figure 3).

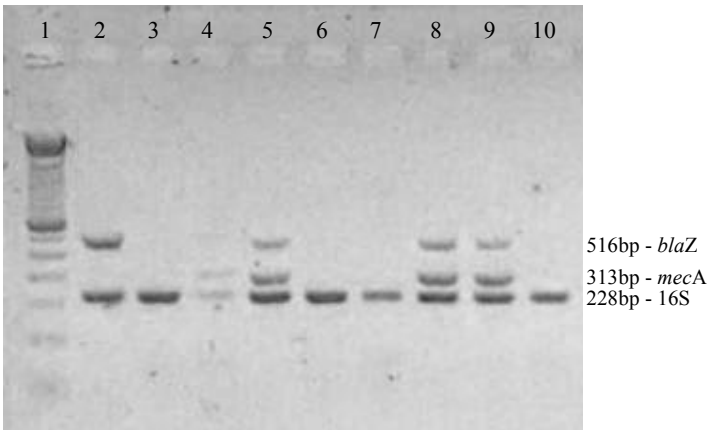

Figure 3. Electrophoresis of agarose gel stained with SYBR® Safe of amplified products of Multiplex PCR for beta-lactam resistance in *Staphylococcus pseudintermedius*. **Line 1**: ladder of 100 bp; **Line 2**: *blaZ*-positive with MIC 0.5 ug/ml; **Line 3**: beta-lactam susceptible with MIC 0.5 ug/ml; **Line 4**: *mec*A-positive with MIC 32 ug/mL; **Line 5**: *mec*A and *blaZ*-positive with MIC 32 ug/mL; **Line 6 and 7**: beta-lactam susceptible with MIC 0.5 ug/ml; **Line 8**: *mec*A and *blaZ*-positive with MIC 64 ug/mL; **Line 9**: *mec*A and *blaZ*-positive with MIC 8 ug/mL; **Line 10**: beta-lactam susceptible with MIC 0.5 ug/ml.

Nineteen (44.19%) of the 46 *Staphylococcus* spp. strains were considered susceptible to β-lactams. Ten (21.74%) strains were carriers of both *mec*A and *bla*Z genes, one (2.17%) carrier *mec*A and *mec*C genes, one (2.17%) only *mec*A gene, and fifteen (32.61%) strains only of the *bla*Z gene (Fig. 4, 5 and 6). None of the 56 samples tested showed the Panton-Valentine leukocidin (PVL) gene.

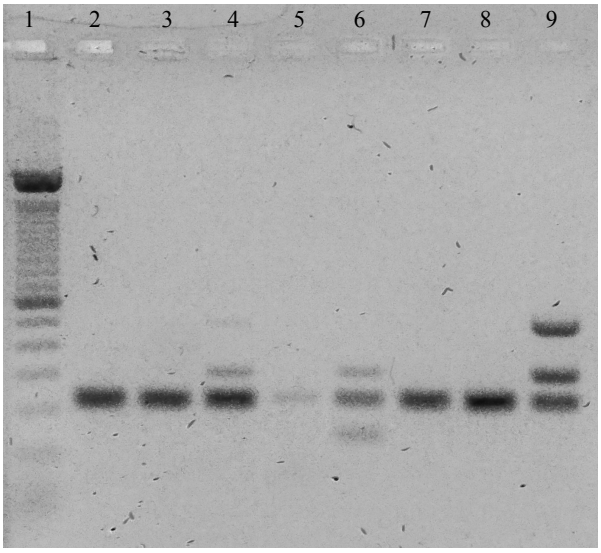

Figure 4. Electrophoresis of agarose gel stained with SYBR® Safe of amplified products of Multiplex PCR for beta-lactam resistance in *Staphylococcus* spp. **Line 1**: ladder of 100 bp; **Line 2, 3, 5, 7 and 8**: beta-lactam susceptible; **Line 4 and 9**: *mec*A and *bla*Z-positive; **Line 6**: *mec*A and *mec*C-positive.

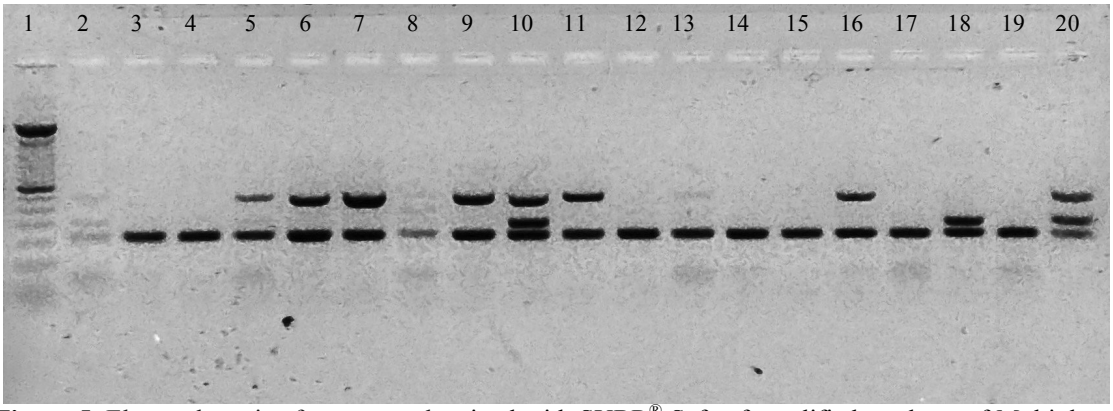

Figure 5. Electrophoresis of agarose gel stained with SYBR® Safe of amplified products of Multiplex PCR for beta-lactam resistance in *Staphylococcus* spp. **Line 1**: ladder of 100 bp; **Line 3, 4, 8, 12, 14, 15, 17 and 19**: beta-lactam susceptible; **Line 2, 10 and 20**: *mec*A and *bla*Z-positive; **Line 18**: *mec*A-positive; **Line 5, 6, 7, 9, 11, 13, 16**: *bla*Z-positive.

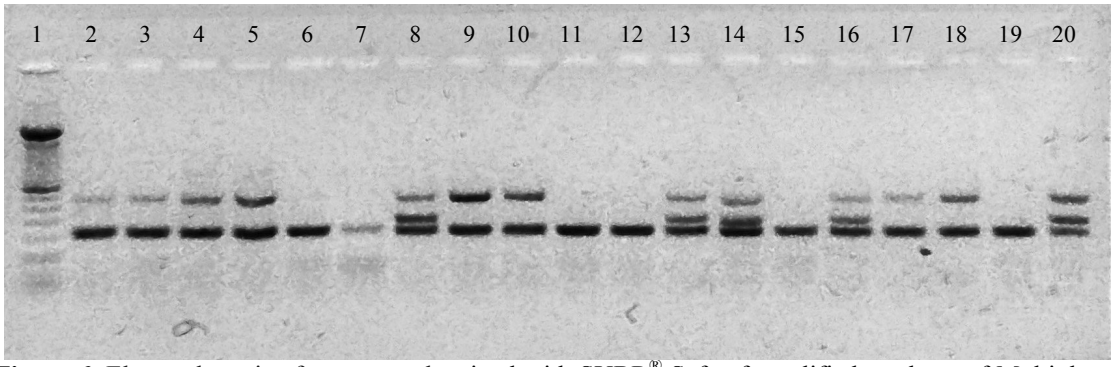

Figure 6. Electrophoresis of agarose gel stained with SYBR® Safe of amplified products of Multiplex PCR for beta-lactam resistance in *Staphylococcus* spp. **Line 1**: ladder of 100 bp; **Line 6, 7, 11, 12, 15 and 19**: beta-lactam susceptible; **Line 8, 13, 14, 16 and 20**: *mec*A and *bla*Z-positive; **Line 2, 3, 4, 5, 9, 10, 17 and 18**: *bla*Z-positive.

DISCUSSION

The important resistance of staphylococci to β-lactam drugs can be generated both by the production of β-lactamase (penicillinase) encoded by the *bla*Z gene (MADIGAN et al., 2010) and by the change in the site of action of β-lactam drugs, mediated by *mec*A or *mec*C (LONCARIC et al., 2019; PATERSON et a., 2014; RUDKIN et al., 2012). The characterization of these two mechanisms of action is necessary for the knowledge and understanding of resistance mechanisms known to β-lactam drugs in all species of staphylococci since several species of staphylococci are implicated in important clinical infections in animals and humans, and these mechanisms of resistance have significant implications ineffective antimicrobial therapy (LONCARIC et al., 2019; TABATABAEI et al., 2019; GUADARBASSI et al., 2013).

The primer design in this study was performed to adjust the hybridization temperature to 54°C by removing bases from 3 primers from Asfour and Darwinsh (2012) and of one primer from Pichon et al. (2012), thus increasing the sensitivity of the technique. The forward *mec*C and reverse PVL primers were entirely designed, using their literature complements, so that the products amplified by each of the genes surveyed had a distinguishable molecular weight for use in the form of multiplex.

Multiplex-PCR reactions are widely used for the identification of β-lactam resistance in *Staphylococcus aureus*. The majority of this works using the identification of *S. aureus* associating with *mec*A gene

(ROCCHETTI et al., 2018; ALI, 2015; LARSEN et al., 2008; RALLAPALLI et al., 2008), including PVL genes (VELASCO et al., 2014), or detecting *S. aureus* associated with the detection of *mec*A and *mec*C genes (BECKER et al., 2013). Stegger et al. (2012) standardized a multiplex-PCR for detection of *mec*A, *mec*C, luk-PV (PVL) and *spa*. Asfour and Darwish (2011) used a multiplex-PCR to detect *mec*A and *blaZ* genes of *Staphylococcus aureus* identified by *nuc* and 16S genes. Picho et al. (2012) developed a multiplex-PCR for detection both *mec*A, *mec*C, and *blaZ*, including *nuc* gene for *S. aureus* detection and PVL gene. Some primers of these authors were used in the present study. However, no studies have been performed to detect any species of *Staphylococcus* associated with the detection of resistance by both the *blaZ* gene and the resistance generated by *mec*A and *mec*C genes, also associating the pathogenicity factor of PVL.

To conclude, the Multiplex PCR described here was able to simultaneously discriminate the *mec*A, *mec*C and *blaZ* genes of β-lactam resistance and PVL gene, a pathogenicity factor, as well as containing the 16S gene amplified product in all samples, assessing in situ all DNA extractions and PCR amplification. This system provides a useful tool for rapid and accurate diagnosis of the two mechanisms of resistance to β-lactam drugs in staphylococci.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

ALI, A.O. Detection of MecA, MecC and Femb Genes by Multiplex Polymerase Chain Reaction. **Journal of Veterinary Advances**, v. 5, n. 12,

2015. doi: 10.5455/jva.20151204111125

ASFOUR, H.A.E.; DARWISH, S.F. Phenotypic and genotypic detection of both *mecA*- and *blaZ*-genes mediated β-lactam resistance in Staphylococcus strains isolated from bovine mastitis. **Global Veterinaria**, v.6, n.1, p.39-50, 2011.

BAUER, A.W.; KIRBY, W.M.M.; SHERRIS, J.C; TURCK, M. Antibiotic susceptibility testing by a standardized single disk method. **American Journal of Clinical Pathology**, v.45, p.493-496, 1966.

BECKER, K.; LARSEN, A.R.; SKOV, R.L.; PATERSON, G.K.; HOMES, M.A.; SABAT, A.J.; FRIEDRICH, A.W.; KÖCK, R.; PETERS, G.; KRIEGESKORTE, A. **Evaluation of a Modular Multiplex-PCR Methicillin-Resistant *Staphylococcus aureus* Detection Assay Adapted for *mecC* Detection. Journal of Clinical Microbiology**, v. 51, n. 6, p. 1917-19, 2013. doi: 10.1128/JCM.00075-13

BRCAST. **Brazilian Commitee on Antimicrobial Suscetibilitty testing**. Tabela pontos de corte clínicos. Julho, 2018.

CLSI. **Performance Standards for Antimicrobial Disk and diluition Susceptibility bactéria isolated from animals**. 3th ed. CLSI document VET01S. Pennsylvania: Clinical and Laboratory Standards Institute; 2015.

CLSI. ***Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals***. 4th ed. CLSI supplement VET08. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.

CLSI. **Performance Standards for Antimicrobial Susceptibility Testing**; Twenty-Third Informational Supplement. CLSI document M100-S23. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; 2013.

GUARDABASSI, L.; LARSEN, J.; WEESE, J.S.; BUTAYE, P.; BATTISTI, A.; KLUYTMANS, J.; LLOYD, D.H.; SKOV, R.L. Public health impact and antimicrobial selection of meticillin-resistant staphylococci in animals. **Journal of Global Antimicrobial Resistance**, v. 1, p. 55-62, 2013. doi: https://doi.org/10.1016/j.jgar.2013.03.011

KANEKO, J.; KAMIO, Y. (2004). Bacterial two-component and hetero-heptameric pore-forming

cytolytic toxins: structures, pore-forming mechanism, and organization of the genes". **Bioscience, Biotechnology, and Biochemistry**, v. 68, n. 5, p. 981–1003, 2004. doi:10.1271/bbb.68.981

LARSEN, A.R.; STEGGER, M.; SØRUM, M. *spa* typing directly from a *mecA, spa* and *pvl* multiplex PCR assay—a cost-effective improvement for methicillin-resistant *Staphylococcus aureus* surveillance. **Clinical Microbiology and Infection**, v. 14, n. 6, p. 611-4, 2008. doi: https://doi.org/10.1111/j.1469-0691.2008.01995.x

LONCARIC, I.; KÜBBER-HEISS, A.; POSAUTZ, A.; RUPPITSCH, W.; LEPUSCHITZ, S.; SCHAUER, B.; FEBLER, A.T.; KRAMETTER-FRÖTSCHER, R.; HARRISON, E.M.; HOMES, M.A.; KÜNZEL, F.; SZOSTAK, M.P.; HAUSCHILD, T.; DESVARS-LARRIVE, A.; MISIC, D.; ROSENGARTEN, R.; WALZER, C.; SLICKERS, P.; MONECKE, S.; EHRICHT, R.; SCHWARZ, S.; SPERGSER, J.; Characterization of mecC gene-carrying coagulase-negative Staphylococcus spp. isolated from various animals. **Veterinary Microbiology**, v. 230, p. 138-144, 2019. doi: https://doi.org/10.1016/j.vetmic.2019.02.014

PATERSON, G.K.; HARRISON, E.M.; HOLMES, M.A. The emergence of *mec*C methicillin-resistant *Staphylococcu aureus*. **Trends in Microbiology**, v. 22, n. 1, p. 42-47, 2014. doi: 10.1016/j.tim.2013.11.003

PICHON, B.; HILL, R.; LAURENT, F.; LARSEN, A.R.; SKOV, R.L.; HOMES, M.; EDWARDS, G.F.; TEALE, C.; KEARNS, A.M. Development a real-time quadruplex PCR assay for simultaneous detection of nuc, PVL, mecA and homologue mecALGA251. **Journal of Antimicrobial Chemotherapy**, v. 67, n. 10, p. 2338–2341, 2012. doi: 10.1093/jac/dks221

RALLAPALLI, S.; VERGHESE, S.; VERMA, R.S. Validation of multiplex PCR strategy for simultaneous detection and identification of methicillin resistant *Staphylococcus aureus.* **PLos One**, v. 9, n. 5, e97617, 2014. doi: 10.1371/journal.pone.0097617

ROCCHETTI, T.T.; MARTINS, K.B.; MARTINS, P.Y.F.; OLIVEIRA, R.A.; MONDELLI, A.L.; FORTALEZA, C.M.C.B.; CUNHA, M.L.R.S. Detection of the *mec*A gene and identification

of *Staphylococcus* directly from blood culture bottles by multiplex polymerase chain reaction. **Brazilian Journal of Infection Disease**, v. 8, n. 2, p. 99-105, 2014. doi: 10.1371/journal.pone.0097617

RUDKIN, J.K.; EDWARDS, A.M.; BOWDEN, M.G.; BROWN, E.L.; POZZI, C.; WATERS, E.M.; CHAN, W.C.; WILIAMS, P.; O'GARA, J.P.; MASSEY, R.C. Methicillin Resistance Reduces the Virulence of Healthcare-Associated Methicillin-Resistant *Staphylococcus aureus* by Interfering With the *agr* Quorum Sensing System. **The Journal of Infectious Diseases,** v.205, n.5, p.798–806, 2012. doi: 10.1093/infdis/jir845

SFACIOTTE, R. A. P.; BORDIN, J. T.; VIGNOTO, V. K. C.; MUNHOZ, P. M.; PINTO, A. A.; BARBOSA, M. J. B.; CARDOZO, R. M.; OSAKI, S. C.; WOSIACKI, S. R. Antimicrobial Resistance in Bacterial Pathogens of Canine Otitis. **American Journal of Animal and Veterinary Sciences**, v.10, n.3, p.162-169, 2015. doi: 10.3844/ajavsp.2015.162.169

SOLANKI, G. Polimerase Chain Reaction. **International Journal of Pharmacological Research**, v. 2, n. 3, 98-102, 2012.

STEGGER, M.; ANDERSEN, P.S.; KEARNS, A.; PICHON, B.; HOLMES, M.A.; EDWARDS, G.; LAURENT, F.; TEALE, C.; SKOV, T.; LARSEN, A.R. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mec*A or the new *mec*A homologue *mec*A_{LGA251}. **Clinical Microbiology and Infection**, v. 18, p. 395-400, 2012. doi: https://doi.org/10.1111/j.1469-0691.2011.03715.x

SZMIGIELSKI, S.; PRÉVOST, G.; MONTEIL, H.; COLIN, D.A.; JELJASZEWICZ, J. Leukocidal toxins of staphylococci. **Zentralbl Bakteriol**. v. 289, n. 2, p. 185–201, 1999. doi:10.1016/S0934-8840(99)80105-4

TABATABAEI, S.; NAJAFIFAR, A.; BADOUEI, M.A.; SAKEHI, T.Z.; TAMAI, I.A.; KHAKSAR, E.; ABBASSI, M.S.; GHAZISAEEDI, F. Genetic characterization of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in pets and veterinary personnel in Iran: new insights into emerging methicillin-resistant *S. pseudintermedius* (MRSP). **Journal of Global Antimicrobial Resistance**, v. 16, p. 6-10, 2019. doi:

https://doi.org/10.1016/j.jgar.2018.08.022

VELASCO, V.; SHERWOOD, J.S.; ROJAS-GARCÍA, P.P; LOGUE, C.M. Multiplex Real-Time PCR for Detection of *Staphylococcus aureus*, *mecA*and Panton-Valentine Leukocidin (PVL) Genes from Selective Enrichments from Animals and Retail Meat. **PLos One**, v. 9, n. 5, e97617, 2014. doi: 10.1371/journal.pone.0097617