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Beta-lactam antimicrobials activity and the diversity of *blaZ* gene in *Staphylococcus aureus* isolates from bovine mastitis in the northwest of Portugal

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ABSTRACT - This study aimed to assess the antimicrobial susceptibility of 52 *Staphylococcus aureus* (*S. aureus*) bovine mastitis isolates obtained from 37 dairy herds from the northwest of Portugal against antibiotics belonging to the β-lactam family, evaluate the detection of *blaZ* and *mecA* resistance genes, and study the diversity of positive isolates. The antimicrobial susceptibility tests were performed by the disk diffusion method. The detection of *blaZ* and *mecA* genes was performed using specific polymerase chain reaction (PCR) and the diversity of *blaZ* was evaluated by phylogenetic analysis. The antimicrobial susceptibility test showed a prevalence of phenotypic resistance by *S. aureus* of 52.0% against penicillin and ampicillin, 34.6% against oxacillin, 17.3% against amoxicillin plus clavulanic acid, and 21.1% against cefazolin. A prevalence of phenotypic intermediate resistance of 5.7% against penicillin, 9.6% against amoxicillin plus clavulanic acid, and 1.9% against ampicillin and cefazolin, respectively, was demonstrated. A 100.0% phenotypic susceptibility was found against piperacillin. Of the 52 *S. aureus* isolates, 35 (67.3%) were PCR positive for *blaZ* gene, and isolate 25 was positive for *mecA* gene. The phylogenetic analysis of partial *blaZ* gene *S. aureus* isolates consensus sequences were placed in two different clades, clade A (cluster A, A.1) and B (cluster B), all closely related to animal and/or human *S. aureus* strains. Isolate 2 appeared in the phylogenetic tree as the most divergent. This study indicates that *blaZ* resistance gene plays a role in β-lactam resistance in the tested bovine mastitis *S. aureus* isolates within dairy herds in the northwest of Portugal, especially in case of penicillin and ampicillin antibiotics that have shown a high phenotypic prevalence. Indeed, the proportion of bovine mastitis isolates with phenotypic resistance did not agree with the proportion of those identified for *blaZ*, as isolates with 100.0% of phenotypic susceptibility for all tested antibiotics also harbored *blaZ*. *BlaZ* phylogenetic analysis from *S. aureus* isolates showed diversity inside or between different herds in the northwest of Portugal. Piperacillin, as a suggestion, could be tested for *S. aureus* bovine mastitis treatment in the future to evaluate this new possibility of therapy.

Keywords: animal production, antibiotic, dairy cattle, disease

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1. Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacteria considered to be an important human pathogen and known as one of the most important agents associated with bovine mastitis worldwide

(Olsen et al., 2006). Bovine mastitis, caused by staphylococcal or other agents, are recognized as an endemic disease and considered as the most prevalent and expensive disease in the dairy farms, still remaining as an economically relevant problem to the dairy industry in several countries (Barkema et al., 2006; Halasa et al., 2007). Several microorganisms, about 140 species, have been recognized as etiological agents of bovine mastitis (Watts, 1988), being coliforms, streptococci, and staphylococci the more often isolated bacteria (Tenhagen et al., 2006; Piepers et al., 2007; Malinowski and Kłossowska, 2010; Smulski et al., 2011). *S. aureus* is of particular importance, because it is highly infectious (Kerro Dego et al**.**, 2002) and is characterized by significantly lower cure levels in comparison with infections caused by other microorganisms (Cramton et al., 1999). Moreover, the *S. aureus* has the potential to expand resistance to almost all the antimicrobial agents (Hiramatsu et al., 2001; Barkema et al., 2009).

β-lactam antibiotics compounds, such as penicillin, continues to be one of the most frequently used drugs in veterinary medicine (Pitkala et al., 2007). Worldwide, antimicrobial resistance by *S. aureus* is extensively spreading following the intensive use of antibacterial agents to treat bovine mastitis. The resistance can be caused by the resistance of the microbe to the antibiotics. On the other hand, biofilms are a survival strategy for bacteria, making them extremely difficult to treat due to their inherent immune response and antibiotic resistance (Peng et al., 2023), as so, the strains that have the ability to form biofilm could cause resistance to the antibiotics, although the strains used in our study were not classified as biofilm producers.

The resistance mechanisms developed by *S. aureus* to β-lactam antibiotics is complex and primarily associated to the *blaZ* (Olsen et al., 2006) and *mecA* genes (Hartman and Tomasz, 1984). The *blaZ* gene is encoded for the β-lactamase enzyme that destroys susceptible β-lactam antibiotics, while *mecA* is the gene encoded for penicillin-binding protein 2a (PBP2a), which is not well inhibited by β-lactams, making cell wall cross-linking possible in bacteria despite the presence of antibiotics (Cha et al., 2007). Both genes are regulated by β-lactam sensor/signal transducer proteins, namely *BlaR1* and *MecR1*, and repressor genes *blaI* and *mecI* (Cha et al., 2007). Furthermore, the detection of *blaZ* is well described in staphylococci from human and cattle origin (Olsen et al., 2006; Asfour and Darwish, 2011) as well as in the case of dogs and cats (Malik et al., 2007).

Additionally, the use of veterinary drugs is sometimes imperative and plays a major role in the control of diseases in cattle populations; a good management and preventive practices in the herds can help the reduction of disease expression and, consequently, the need to resort to drugs that should be done wisely (Falowo and Akimoladun, 2020). In this line, we have recently described the phenotypic characterization and resistance genes detection of *S. aureus* isolated from bovine mastitis in the northwest of Portugal, that could support bovine mastitis control in this region (Hnini et al., 2023).

In this study, we investigated the susceptibility of a set of antibiotics representing all groups of the β-lactam family by the disk diffusion method, against 52 *S. aureus* isolates from bovine mastitis collected from 37 different dairy herds from the northwest of Portugal, in the years 2003-2004, 2007-2008, and 2017. Moreover, the detection by specific PCR methods of the *blaZ* and *mecA* resistance genes was evaluated as well as the phylogenetic analysis of partial *blaZ* gene consensus sequences in selected isolates.

2. Material and Methods

2.1. Samples

Fifty-two *S. aureus* isolates were collected from 37 different dairy herds of the Entre-Douro e Minho region, northwest of Portugal, in the years 2003-2004, 2007-2008, and 2017. The isolates used in this study belonged to the collection of microorganisms of the Laboratory of Microbiology and Infectious Diseases, Department of Veterinary Clinics of the Institute of Biomedical Sciences Abel Salazar of the University of Porto, Porto, Portugal, and SVAExpleite, Lda, Fradelos, Portugal.

2.2. Antimicrobial susceptibility test

The antimicrobial susceptibility testing was performed in all *S. aureus* isolates by the disk diffusion method following guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2007; CLSI, 2014). Isolates streaked on Columbia ANC agar supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) were grown overnight at 37 °C. Afterward, colonies were re-suspended in 1 mL of 0.85% (w/v) sodium chloride (Merck Laboratories, Darmstadt, Germany) and adjusted to 0.5 McFarland in comparison with a McFarland standard (bioMérieux, Marcy l'Etoile, France). Then, Mueller–Hinton agar (Merck Laboratories, Darmstadt, Germany) plates were inoculated with the inoculum by dipping sterile cotton swabs into the bacterial suspension. Then, antibiotic disks from groups of the β-lactam family, such as Penicillin G (Penicillin (10 U)), Penicillin M (Oxacillin (1 µg)), Aminopenicillins (Ampicillin (10 µg), Amoxicillin plus Clavulanic acid (20 µg + 10 µg)), Ureidopenicillin (Piperacillin (100 µg)) and first-generation Cephalosporin (Cefazolin (30 µg)) (all from bioMérieux, Marcy l'Etoile, France), were applied to the inoculated plates and incubated at 37 °C for 24 h. After the incubation period, diameters of the inhibition zones were measured in millimeters and compared with the ranges suggested by the CLSI guidelines. The isolates were classified on the basis of the size of the inhibition zone, following definitions: resistant – bacteria are *in vitro* inhibited by a concentration of an antimicrobial agent that is associated with a high probability of therapeutic failure; intermediate – bacteria are *in vitro* inhibited by a concentration of an antimicrobial agent that is associated with an uncertain therapeutic effect; and susceptible – bacteria are *in vitro* inhibited by a concentration of an antimicrobial agent that is associated with a high probability of therapeutic success (Nabal Díaz et al., 2022). Test performance was monitored using *S. aureus* ATCC 29213 strain.

2.3. DNA extraction

Two colonies of each staphylococcal isolate previously streaked onto Columbia ANC agar supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) were inoculated in tubes with 10 mL of BHI and incubated at 37 °C for 24 h. Afterward, tubes were centrifuged at 10000 × *g* for 10 min, and genomic DNA was extracted from pellets using the QIAamp® DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer instructions.

2.4. PCR of *blaZ* and *mecA* resistance genes and sequencing

Polymerase chain reactions targeting *blaZ* and *mecA* genes were performed accordantly as previously described (Olsen et al., 2006; Szweda et al., 2014). Briefly, after DNA extraction, all isolates were tested for *blaZ* and *mecA* using the set of primers 487 (5′-TAAGAGATTTGCCTATGCTT-3′) / 373 (5′-TTAAAGTCTTACCGAAAGCAG-3′) and *mecA*fw (5′-AAAATCGATGGTAAAGGTTGG-3′) / *mecA*rev (5′-AGTTCTGCAGTACCGGATTTGC-3′), respectively, by PCR in a thermocycler C 1,000 (Bio-Rad, California, USA). The amplified products (*blaZ*-377bp and *mecA*-533bp) were analyzed on a 1.5% (w/v) agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Duren, Germany) and visualized under ultraviolet light (Bio-Rad, California, USA). Following the amplification, 32 *blaZ* gene amplicons, selected for sequencing, were purified with the NZYGelpure kit (nzytech, Lisbon, Portugal) and directly sequenced at GATC Biotech (Cologne, Germany) using the same primers. Retrieved sequences were analyzed, and a consensus sequence for each isolate was created after overlapping of the obtained sequences from forward and reverse primers using MEGA version 5.0 (Kimura, 1980). All sequences were deposited on GenBank database under accession numbers KY020052 (isolate 1, herd 1), KY020053 (isolate 2, herd 1), KY020054 (isolate 3, herd 1), (isolate 7, herd 5), KY020059 (isolate 8, herd 6), KY020060 (isolate 9, herd 7), KY020061 (isolate 10, herd 8), KY020062 (isolate 11, herd 8), KY020063 (isolate 12, herd 9), KY020064 (isolate 13, herd 10), KY020065 (isolate 14, herd 11), KY020066 (isolate 15, herd 12), KY020067 (isolate 16, herd 13), KY020068 (isolate 17, herd 14), KY020069 (isolate 18, herd 15), KY020070 (isolate 19, herd 16), KY020071 (isolate 20, herd 17), KY020072 (isolate 21, herd 18), KY020073 (isolate 22, herd 19), KY020074 (isolate 23, herd 20), KY020075 (isolate 24, herd 20), KY020076 (isolate 25, herd 21),

KY020077 (isolate 26, herd 22), MH350414 (isolate 36, herd 28), MH350415 (isolate 38, herd 29), MH350416 (isolate 39, herd 29), MH350417 (isolate 40, herd 29), MH350418 (isolate 41, herd 30), and MH350419 (isolate 50, herd 36).

2.5. Phylogenetic analysis

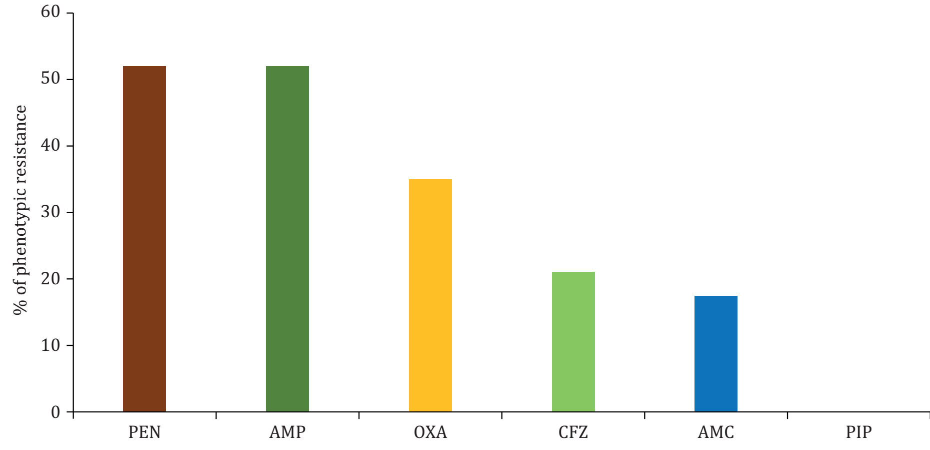
The generated partial amino acid sequences of *blaZ* gene were aligned using Clustal W through MEGA version 5.0 (Kimura, 1980) for phylogenetic inference. An evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−781.1857) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 200.0000). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 61 amino acid sequences. All positions containing gaps and missing data were eliminated. There were 79 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0 (Kimura, 1980).

For the phylogenetic analysis, the consensus sequences were aligned with human, bovine, and animal food *S. aureus* sequences, animal *S. warneri*, and *S. intermedius* sequences, and with two *S. haemolyticus* sequences, one from air and the other from human/animal origin that was used as an outgroup. For all selected strains, respective Genbank accession number is referred in the phylogenetic tree.

3. Results

3.1. Phenotypic assessment of β-lactam antimicrobials

The antimicrobial susceptibility test for the tested 52 *S. aureus* isolates demonstrated a prevalence of phenotypic susceptibility of 100.0% (n = 52) to piperacillin (Figure 1 and Table 1). Furthermore, a prevalence of phenotypic resistance of 52.0% (n = 27) was demonstrated to penicillin and ampicillin, 34.6% (n = 18) to oxacillin, 17.3% (n = 9) to amoxicillin plus clavulanic acid, and 21.1% (n = 11) to cefazolin (Figure 1 and Table 1).



PEN - penicillin; AMP - ampicillin; OXA - oxacillin; CFZ - cefazoline; AMC - amoxicillin plus clavulanic acid. PIP - piperacillin.
Percentages were used for descriptive analysis in Microsoft® Excel® 2016 MSO.

Figure 1 - Phenotypic resistance percentage of the 52 *S. aureus* isolates achieved against the β-lactams antimicrobials tested in this study.

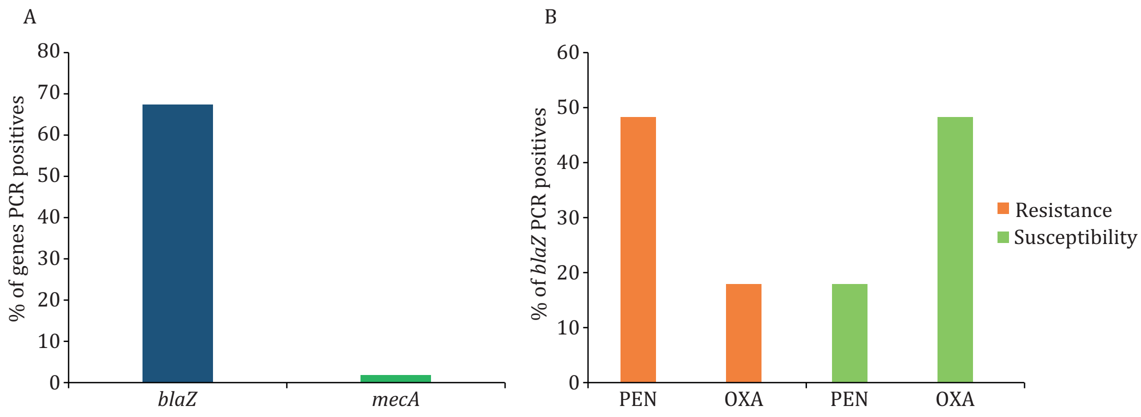
Table 1 - Antimicrobial tests performed to all *S. aureus* isolates from bovine mastitis against β-lactam antibiotics by disk diffusion method following CLSI guidelines

Isolate	β-lactam antibiotics in disks					
	PEN (10 U)	AMP (10 µg)	OXA (1 µg)	AMC (20 µg + 10 µg)	CFZ (30 µg)	PIP (100 µg)
1	R	R	S	S	S	S
2	S	S	S	S	S	S
3	R	R	S	S	S	S
4	R	R	S	S	S	S
5	R	R	S	S	S	S
6	R	R	S	S	S	S
7	R	R	S	S	S	S
8	R	R	S	S	S	S
9	R	R	S	S	S	S
10	R	R	S	S	S	S
11	R	R	S	S	S	S
12	S	S	S	S	S	S
13	R	R	S	S	S	S
14	R	R	S	S	S	S
15	R	R	S	S	S	S
16	R	R	S	S	S	S
17	R	R	S	S	S	S
18	R	R	S	S	S	S
19	R	S	S	S	S	S
20	R	S	S	S	S	S
21	S	S	S	S	S	S
22	R	R	S	S	S	S
23	S	S	S	S	S	S
24	S	S	S	S	S	S
25	R	R	R	S	S	S
26	S	S	S	S	S	S
27	S	S	R	I	I	S
28	S	S	I	I	S	S
29	R	R	R	R	R	S
30	S	S	S	S	S	S
31	S	S	S	S	S	S
32	S	S	S	S	S	S
33	S	S	R	I	S	S
34	S	S	S	S	S	S
35	R	R	R	I	R	S
36	I	R	R	R	R	S
37	S	S	R	R	R	S
38	R	R	R	R	R	S
39	R	R	R	R	R	S
40	R	R	R	R	R	S
41	I	R	R	R	R	S
42	S	S	S	S	S	S
43	S	S	R	S	S	S
44	S	S	S	S	S	S
45	S	S	S	S	S	S
46	R	R	R	R	R	S
47	S	S	S	S	S	S
48	S	S	R	S	S	S
49	S	S	R	S	S	S
50	R	R	R	R	R	S
51	S	S	R	S	S	S
52	I	I	R	I	R	S

CLSI - Clinical and Laboratory Standards Institute; S - susceptible; I - intermediate; R - resistant; PEN - penicillin; AMP - ampicillin; OXA - oxacillin; AMC - amoxicillin + gluvalunic acid; CFZ - cefazolin; PIP - piperacillin.

3.2. Presence of the *blaZ* and *mecA* resistance genes

Among all tested *S. aureus* isolates (n = 52), 67.3% (n = 35) and 1.9% (n = 1) were *blaZ* and *mecA* PCR positives, respectively (Table 2 and Figure 2). Furthermore, within the *blaZ* PCR results, 48.1% (25/52) and 17.3% (9/52) have phenotypic resistance against penicillin and oxacillin, respectively, and the same values (same antimicrobials, similar or different isolates) were achieved for phenotypic susceptibility (Table 2 and Figure 2). Moreover, isolate 25 (*mecA* PCR positive) has phenotypic resistance to penicillin and oxacillin (Table 2 and Figure 2).



A: Percentage of detection of *blaZ* and *mecA* resistance genes. B: Percentage of detection of penicillin (PEN) and oxacillin (OXA) resistance and susceptibility within *blaZ* gene.
Percentages were used for descriptive analysis in Microsoft® Excel® 2016 MSO.

Figure 2 - Detection of the *BlaZ* and *mecA* resistance genes among the 52 *S. aureus* isolates in this study by PCR, and PEN and OXA resistance and susceptibility within the *blaZ* gene.

3.3. Sequencing analysis of *blaZ*

Thirty-two positive isolates were selected for sequencing partial *blaZ* gene. The retrieved sequences were analyzed, and a consensus sequence for each isolate was created. When blastn of nucleotide consensus sequences were conducted in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), similarities of 100.0-99.0% and e-values of 0.0-5e-158 were shared for *S. aureus* strains (Table 3). This data supported the selection of the *S. aureus* strains used in the phylogenetic analysis.

3.4. Phylogenetic analysis of *blaZ*

Phylogenetic relationships were inferred using the ML method as described in the Material and Methods section. The phylogenetic analysis placed the 32 *S. aureus* isolates in two different clades, clade A and B, supported by bootstrap values of 76.0 and 93.0% (of 1,000 replicates), respectively (Figure 3). The isolates 1, 3-13, 19-20, and 22-26 were placed in clade A, supported by bootstrap values of 76.0% (of 1,000 replicates), and are closely related to bovine and human *S. aureus* strains (Figure 3). Moreover, within clade A, there is a cluster A with a sub-cluster A.1, supported by bootstrap values of 85.0 and 73.0% (of 1,000 replicates), respectively. Isolate 21 is placed as single within cluster A, being the most divergent within cluster A, and isolate 14 is placed in the sub-cluster A.1, closely related to a human *S. aureus* strain (Figure 3). Relatively to clade B, isolates 15-18 are placed more closely related to human *S. aureus* strains, and within cluster B, isolates 36, 38-41, and 50 appeared placed more closely related to animal food, animal, and human *S. aureus* strains (Figure 3). Lastly, isolate 2 appeared in the phylogenetic tree as the most divergent of all analyzed *S. aureus* strains in this study (Figure 3).

Table 2 - PCR tests performed to all *S. aureus* isolates from bovine mastitis with herds and year of collection

Herd	Isolate	Year of sample collection	*BlaZ* gene	*mecA* gene
1	1	2003	+	–
1	2	2008	+	–
1	3	2008	+	–
2	4	2003	+	–
3	5	2004	+	–
4	6	2003	+	–
5	7	2003	+	–
6	8	2003	+	–
7	9	2004	+	–
8	10	2003	+	–
8	11	2003	+	–
9	12	2003	+	–
10	13	2003	+	–
11	14	2003	+	–
12	15	2003	+	–
13	16	2003	+	–
14	17	2003	+	–
15	18	2003	+	–
16	19	2004	+	–
17	20	2007	+	–
18	21	2008	+	–
19	22	2008	+	–
20	23	2008	+	–
20	24	2008	+	–
21	25	2008	+	+
22	26	2008	+	–
23	27	2017	+	–
23	28	2017	+	–
23	29	2017	–	–
24	30	2017	–	–
24	31	2017	–	–
25	32	2017	–	–
25	33	2017	–	–
26	34	2017	–	–
27	35	2017	+	–
28	36	2017	+	–
29	37	2017	+	–
29	38	2017	+	–
29	39	2017	+	–
29	40	2017	+	–
30	41	2017	–	–
30	42	2017	–	–
31	43	2017	–	–
31	44	2017	–	–
31	45	2017	–	–
32	46	2017	–	–
33	47	2017	–	–
34	48	2017	–	–
35	49	2017	–	–
36	50	2017	+	–
37	51	2017	–	–
37	52	2017	–	–

+ Positive; – negative.

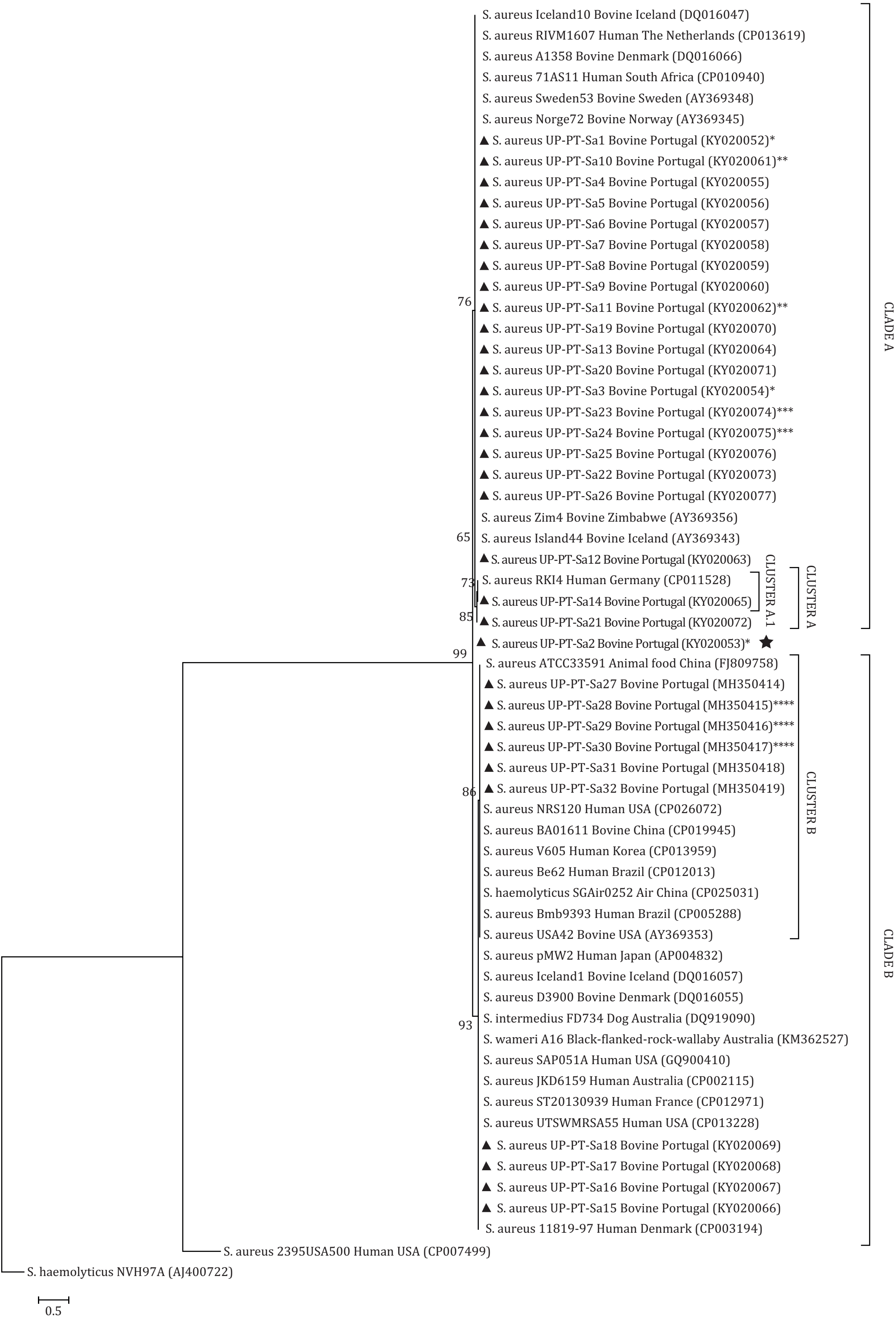
Table 3 - Blastn between consensus sequences of all tested bovine mastitis *S. aureus* isolates in the NCBI-GenBank database[1]

Isolate	GenBank accession no.	Strain	Position	E-value	% Identities
1	CP013619	*Staphylococcus aureus* strain RIVM1607	2008502 - 2008879	0.0	100.0
2	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	99.0
3	CP013619	*Staphylococcus aureus* strain RIVM1607	2008502 - 2008865	0.0	100.0
4	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
5	CP013619	*Staphylococcus aureus* strain RIVM1607	2008502 - 2008879	0.0	99.0
6	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	99.0
7	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
8	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
9	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
10	DQ016066	*Staphylococcus aureus* strain A1358_1	181 - 544	0.0	100.0
11	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
12	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	99.0
13	DQ016066	*Staphylococcus aureus* strain A1358_1	181 - 543	0.0	100.0
14	CP011528	*Staphylococcus aureus* strain RKI4	1929299 - 1929676	0.0	99.0
15	CP012971	*Staphylococcus aureus* strain ST20130939	13433 - 13797	0.0	99.0
16	CP012971	*Staphylococcus aureus* strain ST20130939	13433 - 13797	0.0	99.0
17	CP012971	*Staphylococcus aureus* strain ST20130939	13420 - 13797	0.0	99.0
18	CP012971	*Staphylococcus aureus* strain ST20130939	13420 - 13797	0.0	99.0
19	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
20	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
21	CP011528	*Staphylococcus aureus* strain RKI4	1929299 - 1929676	0.0	99.0
22	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
23	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
24	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	99.0
25	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
26	DQ016066	*Staphylococcus aureus* strain A1358_1	168 - 544	0.0	100.0
36	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 - 2886572	5e-158	100.0
38	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 - 2886572	5e-158	100.0
39	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 - 2886572	5e-158	100.0
40	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 - 2886572	5e-158	100.0
41	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 - 2886572	5e-158	100.0
50	LR130515	*Staphylococcus aureus* strain BPH2947 genome assembly, chromosome: 1	2886266 - 2886572	5e-158	100.0

[1] https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch

4. Discussion

The results obtained from the antimicrobial susceptibility testing showed a resistance prevalence of 52.0% to penicillin (Penicillin G group), followed by 52.0% to ampicillin (Aminopenicillin group), and 34.6, 21.1, and 17.3% for oxacillin (Penicillin M group), cefazolin (Cephalosporin group), and amoxicillin plus clavulanic acid (Penicillin M group), respectively. The studies on resistance by *S. aureus* causing bovine mastitis in Portugal are scarce; nevertheless, a moderate to high prevalence of resistance to penicillin (66.7%, n = 20) was described in the central region of Portugal (Nunes et al., 2007) when compared with that found in this study (52.0%), meaning that a lower prevalence was shown here.



ML method was inferred. Bootstrap resampling was used to determine the robustness of branches; values from 1,000 replicates are shown.
Filled triangle indicates the 52 bovine mastitis *S. aureus* isolates tested in this study.
* Isolates from herd 1; ** Isolates from herd 8; *** Isolates from herd 20; **** Isolates from herd 23.

Figure 3 - Phylogenetic analysis of *S. aureus blaZ* gene sequences.

However, resistance against β-lactam antibiotics especially penicillin, ampicillin, and amoxicillin were described in numerous different geographical regions of the world. Klimienė et al. (2011) showed different rates of penicillin resistance (76.7%) with a high increased level for ampicillin (78.4%) and amoxicillin (81.3%) in Lithuania in comparison with our study, suggesting that amoxicillin appeared less effective against bovine mastitis by *S. aureus* strains in Lithuania than in Portuguese dairy herds. Furthermore, a prevalence resistance to bovine mastitis *S. aureus* strains for amoxicillin, ampicillin, and penicillin of 20.6 and 36.0% was described in New Zealand and in the United States of America, respectively (Petrovski et al., 2015). Moreover, isolates with a higher resistance prevalence (87.2%) in comparison with those found in our study (52.0%) were previously described in Africa (South West Ethiopia) (Sori et al., 2011), and an almost equal prevalence rate of resistance was described in Argentinian dairy herds (48.4%) (Russi et al., 2008). Here, an oxacillin resistance prevalence of 34.6% was found, while the same authors described 0.0% (Russi et al., 2008).

Nevertheless, a phenotypic resistance to oxacillin of 12.8% was previously described in *Staphylococcus* spp. including *S. aureus* isolated from small ruminant mastitis in Brazil (França et al., 2012). Here, cefazolin demonstrated an antimicrobial resistance of 21.1% against tested *S. aureus*, and an antimicrobial resistance of 33.3% in *S. aureus* isolates obtained from clinical and sub-clinical mastitis was previously described (Sharma et al., 2015). Finally, piperacillin (Ureidopenicillin group) within the β-lactams group was also evaluated in this study, and 100.0% of *in vitro* activity against tested *S. aureus* isolates was shown. There are few studies focusing on this antimicrobial, while a high antimicrobial sensitivity against *S. aureus* isolates obtained from clinical and sub-clinical mastitis against piperacillin plus tazobactam (88.9%) was previously described (Sharma et al., 2015). In this line, we suggest that piperacillin could be tested for *S. aureus* bovine mastitis treatment in the future to evaluate this new possibility of therapy. The overall findings could be associated to the drug selection used for treatment in each country, as the choice of antimicrobials to be applied will depend on the local availability and respective regulations.

Relatively to the *blaZ* and *mecA* resistance genes tested among all *S. aureus* bovine mastitis in this study, 67.3% of them were positive for the *blaZ* and negative for the *mecA* genes, except for isolate 25 that was positive for *mecA*. The resistance mechanisms developed by *S. aureus* to β-lactam antibiotics is complex and primarily associated to the *blaZ* (Olsen et al., 2006) and *mecA* genes (Hartman and Tomasz 1984). Nevertheless, here the antimicrobial resistance shown against tested β-lactam antibiotics suggests a greater association linked to the *blaZ* gene. Interestingly, isolate 25, positive for *mecA* gene, was found to be resistant to oxacillin, the antibiotic suggested to detect methicillin resistance instead of methicillin during our study (Cunha, 2005). Additionally, nowadays, cefoxitin is recommended for this evaluation instead of oxacillin. The resistance of *S. aureus* to oxacillin, due to the acquisition of the *mecA* gene, has been previously described (Al-Akydy et al., 2014). There are numerous mechanisms of antimicrobial resistance to β-lactam antibiotics, and one of the most important is associated with the production of β-lactamases (Bush et al., 1995; McManus, 1997; Holten and Onusko, 2000). While in a specific region, other factors can be involved in the resistance against a particular antibiotic, it can be due to the frequent and long-term antibiotic utilization (Sabour et al., 2004; Moon et al., 2007; Kumar et al., 2010a; Kumar et al., 2010b; Sharma et al., 2015).

Resistance has also been associated to *S. aureus* biofilm strains producers, and the process of biofilm formation is complex and involves the co-expression of multiple genes (Peng et al., 2023), while the strains in our study were not classified as biofilm producers. In other published studies on this topic, it appeared that the prevalence of resistance to antibiotics (penicillin and ampicillin) is higher in bovine mastitis caused by *S. aureus* (Li et al., 2009). Furthermore, in most cases, authors have associated the high rates of resistance encountered in bovine mastitis *S. aureus* strains with the production of β- lactamase encoded by the gene *blaZ* (Watts and Salmon, 1997; Szweda et al., 2014).

In general, the penicillin resistance presented by *S. aureus* is conferred by two well-known mechanisms. One mechanism, considered to be the most important, is directly associated with the expression of the enzyme β-lactamase, which can hydrolyze the antibiotic, rendering it inactive (Hartman and Tomasz, 1984). The other mechanism, primarily related with human isolates, is

responsible to the resistance linked to the PBP2a protein, encoded by *mecA* gene, and plays a role in methicillin resistance, which is a much less sensitive target than the wild-type PBPs (Hartman and Tomasz, 1984; Deurenberg et al., 2007).

In the present study, there are six *S. aureus* isolates (2, 12, 21, 23, 24, 26) with phenotypic susceptibility to all tested antibiotics that also harbor *blaZ* resistance gene (Tables 1 and 2). To our knowledge, there is not enough data explaining this phenomenon, but Haveri et al. (2005) suggested that phenotypically susceptible isolates that carry resistance genes should be considered as potentially resistant. Furthermore, besides detecting the gene, further studies on the evaluation of the gene expression/unexpression must be done to better explain this phenomenon, as *blaZ* is regulated by β-lactam sensor/signal transducer proteins, BlaR1, and repressor BlaI (Cha et al., 2007).

Moreover, the development of a novel heterologous β-lactam-specific whole-cell biosensor in *Bacillus subtilis*, based on the β-lactam-induced regulatory system BlaR1/BlaI from *S. aureus*, was previously described (Lautenschläger et al., 2020; Tasara et al., 2013). Reported data showed that, among 10 *S. aureus* strains carrying *blaZ* gene, five strains were phenotypically resistant to penicillin while the other five (all belonging to the clonal complex 8) were susceptible to penicillin (Tasara et al., 2013). The presence of the *blaZ* in all five strains were confirmed by PCR, while the sequencing results of these genes uncovered a 29-base deletion within the *blaZ* gene in all these strains that cause a translational frame shift, which is predicted to induce abrogation of *blaZ* expression (Tasara et al., 2013).

On the other hand, the possibility of incorrect procedure in the antimicrobial testing performance was out of question, as all tests were performed equally and with achieved repeatable different results. Here, different sequences were recovered from the nine *S. aureus* tested isolates and they were phylogenetically placed in different clusters, disrupting the possibility of contamination during the *blaZ* PCR procedure. The *blaZ* gene phylogenetic analysis placed the 32 *S. aureus* isolates selected for sequencing in two different clades, clade A and B, and they are closely related to different bovine mastitis and/or human *S. aureus* strains. The study involved isolates from bovine mastitis samples collected in the years of 2003, 2004, 2007, 2008, and 2017 in herds geographically nearby, and as expected, there was a phylogenetic divergence of analyzed strains observed during this period of time.

Almost all isolates belonging to the years of collection 2003, 2004, 2007, and 2008 are placed in the clade A, which appeared closely related to animal and human *S. aureus* strains. However, isolates 15, 16, 17, and 18 (2003) appeared placed in the clade B with the recent (2017) *S. aureus* isolates, all also related with animal and human *S. aureus* strains. To note, and as expected, the recent isolates appeared phylogenetic separately from the 2003 *S. aureus* strains in the clade B, being genetically related between them, but phylogenetically divergent from the 2003 *S. aureus* strains. Furthermore, isolate 2 (collected in 2003, from herd 1 as isolates 1 (2003) and 3 (2008)), appeared placed single in the phylogenetic tree, being the most phylogenetically divergent strain in this study, and, even though it is a "relatively older" *S. aureus* strain, it may be interesting to deepen it genetically in the future.

In addition, the older isolates in this study (mainly placed in clade A) are related to a high prevalence of penicillin and ampicillin resistance, and the recent ones (placed in clade B) are related with a higher prevalence of resistance to almost all tested antibiotics (penicillin, ampicillin, oxacillin, amoxicillin plus clavulanic acid, and cefazolin), suggesting that this is in the line of evolution/divergence of the strains in the testing period of time, by the use, or the excess use of antibiotics in animal treatments. We were able to detect and sequence *blaZ* gene according to the methods previously described (Olsen et al., 2006); however, the evaluated diversity in this study, as described above, was not done in the context of chromosome or plasmid gene location.

5. Conclusions

This study indicates that *blaZ* resistance gene plays a role in β-lactam resistance in the tested bovine mastitis *S. aureus* isolates within dairy herds in the northwest of Portugal, especially in case of penicillin and ampicillin antibiotics that have shown a high phenotypic prevalence. However, the

proportion of bovine mastitis isolates with phenotypic resistance did not agree with the proportion of those identified for *blaZ*, as isolates with 100.0% of phenotypic susceptibility for all tested antibiotics also harbored *blaZ.* Finally, *blaZ* phylogenetic analysis from *S. aureus* isolates showed diversity inside or between different herds in the northwest of Portugal. The evaluation of new bovine mastitis milk samples collected in the same herds, using the same or other methods, would be of importance to further discuss the dynamics on resistance patterns of *S. aureus* in the region. Piperacillin, as a suggestion, could be tested for *S. aureus* bovine mastitis treatment in the future to evaluate this new possibility of therapy.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Hnini, R.; Silva, E. and Thompson, G. **Data curation:** Hnini, R.; Silva, E. and Pinho, L. **Formal analysis:** Hnini, R. and Silva, E. **Software:** Hnini, R. and Silva, E. **Supervision:** Silva, E.; Najimi, M. and Thompson, G. **Validation:** Hnini, R.; Silva, E.; Pinho, L.; Najimi, M. and Thompson, G. **Writing – original draft:** Hnini, R. and Silva, E.

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