Beta-lactamase detection in *Staphylococcus aureus* and coagulase-negative *Staphylococcus* isolated from bovine mastitis[1]

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ABSTRACT.- Robles B.F., Nóbrega D.B., Guimarães F.F., Wanderley G.G. & Langoni H. 2014. **Beta-lactamase detection in *Staphylococcus aureus* and coagulase-negative *Staphylococcus* isolated from bovine mastitis.** *Pesquisa Veterinária Brasileira 34(4):325-328.* Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Distrito de Rubião Junior s/n, Botucatu, SP 18618-900, Brazil. E-mail: hlangoni@fmvz.unesp.br

The objectives of the study were to evaluate the presence/production of beta-lactamases by both phenotypic and genotypic methods, verify whether results are dependent of bacteria type (*Staphylococcus aureus* versus coagulase-negative *Staphylococcus* - CNS) and verify the agreement between tests. A total of 200 bacteria samples from 21 different herds were enrolled, being 100 CNS and 100 *S. aureus.* Beta-lactamase presence/detection was performed by different tests (PCR, clover leaf test - CLT, Nitrocefin disk, and in vitro resistance to penicillin). Results of all tests were not dependent of bacteria type (CNS or *S. aureus*). Several *S. aureus* beta-lactamase producing isolates were from the same herd. Phenotypic tests excluding in vitro resistance to penicillin showed a strong association measured by the kappa coefficient for both bacteria species. Nitrocefin and CLT are more reliable tests for detecting beta-lactamase production in staphylococci.

INDEX TERMS: Beta-lactamase, *blaZ*, coagulase-negative *Staphylococcus*, *Staphylococcus aureus*.

RESUMO.- [**Detecção de beta-lactamase em *Staphylococcus aureus* e *Staphylococcus* coagulase negativa isolados de mastite bovina.**] Os objetivos do presente estudo foram avaliar a presença/produção de beta-lactamases por ambos os métodos fenotípicos e genotípicos, verificar se os resultados são dependentes do tipo de bactéria (*Staphylococcus aureus* contra *Staphylococcus* coagulase negativa - CNS) e verificar a concordância entre os testes. Um total de 200 amostras bactérianas oriundas de 21 rebanhos distintos foram incluídos, sendo 100 CNS e 100 *S. aureus*. A presença/detecção de beta-lactamase foi realizada por diferentes testes (PCR, teste trevo (*clover leaf test*) - CLT, disco Nitrocefin e resistência in vitro à penicilina). Os resultados de todos os testes não foram dependentes do tipo de bactérias (CNS ou *S. aureus*). Vários isolados de *S. aureus* produtores de beta-lactamase eram de um mesmo rebanho. Testes fenotípicos excluindo resistência in vitro à penicilina mostraram uma forte associação medida pelo coeficiente kappa para ambas as espécies de bactérias. Nitrocefina e CLT são testes mais confiáveis para detectar a produção de beta-lactamase em estafilococos.

TERMOS DE INDEXAÇÃO: Beta-lactamase, *bla*Z, *Staphylococcus* coagulase negativa, *Staphylococcus aureus.*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the most common causes of contagious bovine mastitis (Melchior et al. 2006). Coagulase-negative *Staphylococcus* (CNS) are commonly isolated from mastitis cases in several countries (Pitkala et al. 2004, de Freitas Guimarães et al. 2013), with limited knowledge and published papers regarding antimicrobial resistance mechanisms. In recent years, treatment of CNS-caused infections has become an important topic. As *S. aureus,* CNS can harbor resistance genes to several antimicrobials (Hammad et al. 2012, Silva et al. 2013).

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Beta-lactam compounds such as penicillin continues to be one of the most frequently used drugs in veterinary medicine (Pitkala et al. 2007). Two primary resistance mechanisms to beta-lactams are noteworthy in *Staphylococcus* spp.: the expression of beta-lactamase enzymes encoded by the *blaZ* gene, and production of the penicillin-binding protein 2a resulting in a higher-level of resistance encoded by the *mecA* gene (Fuda et al. 2005). Prevalence of penicillin resistance in staphylococci causing animal diseases is most commonly due to the *blaZ* gene (Malik et al. 2007, Pitkala et al. 2007).

Different tests can be performed to evaluate beta-lactamase production in staphylococci. A qualitative procedure for detecting production of beta-lactamase is the usage of Nitrocefin disks. The reaction is based on the production of a colored compound when the substrate (nitrocefin) is exposed to a beta-lactamase-producing bacteria. The clover leaf test (CLT) is an alternative with high sensitivity and specificity for investigating beta-lactamase production in staphylococci (Bergan et al. 1997).

Studies regarding beta-lactamase production in *Staphylococcus* isolates and comparison of tests are scarce. Comparison of beta-lactamase activity in coagulase positive and negative isolates would prove to be valuable, adding more results to available literature.

The objectives of the present study were to evaluate the presence/production of beta-lactamases by both phenotypic and genotypic methods, verify whether results are dependent of bacteria type (coagulase positive versus coagulase negative *Staphylococcus*) and verify the agreement between tests. Our hypotheses were that more coagulase-positive *Staphylococcus* isolates would present beta-lactamase enzymes and tests would have a high agreement coefficient.

MATERIALS AND METHODS

Samples. A total of 200 bacteria samples from 21 different herds located in São Paulo State were enrolled, being 100 CNS and 100 *Staphylococcus aureus* (Table 1). Isolates were taken from cows with diagnosed intramammary infection (IMI) from 2009 to 2012 and stored at -80°C.

Microbiological procedures. In vitro antimicrobial susceptibility testing was conducted by disc diffusion method using the two following antimicrobial agents: oxacillin and penicillin (Oxoid Ltd., Basingstoke, Hampshire, England), and interpreted according to the Clinical and Laboratory Standards Institute (CLSI 2008). Beta-lactamases detection was performed by three methods.

Nitrocefin® Disks. Nitrocefin® commercial disks (Becton Dickinson Microbiology Systems, Cockeysville, United States) were acquired and stocked at -10° C until usage. Disks were initially embedded in saline solution, and with a sterile loop, colonies were streaked onto its surface. Disks were observed within 60 minutes at ambient temperature. A positive reaction was considered as a change of colour from yellow to pink. *S. aureus* ATCC 29213 and *S. aureus* 25923 were used as positive and negative control respectively.

Clover Leaf Test (Hodge Test). The clover leaf test was performed according to Bergan et al. (1997). Presence of an irregular inhibition zone was considered as a positive result. *S. aureus* ATCC 29213 and *S. aureus* 25923 were used as positive and negative control respectively.

PCR detection of *blaZ* gene. PCR reactions aiming the *blaZ* gene were performed with primers blaZf (5 'AAGAGATTTGCCTATGCTTC 3) and blaZr (5' GCTTGACCACTTTTATCAGC 3') (Haveri et al. 2005). Extraction and purification were performed by physical methods (boiling and centrifugation) (Malik et al. 2007). *S. aureus* ATCC 29213 and *S. aureus* 25923 were used as positive and negative control respectively.

Table 1. Absolute (n), relative (%) and cumulative (Cf) frequencies of coagulase-negative *Staphylococcus* (CNS) and *Staphylococcus aureus* isolates enrolled in the present study according to herd number

Bacteria	Herd	N	%	Cf	Bacteria	Herd	N	%	Cf
CNS	1	11	11	11	*S. aureus*	1	-	-	-
	2	-	-	-		2	14	14	14
	3	6	6	17		3	4	4	18
	4	-	-	-		4	1	1	19
	5	2	2	19		5	1	1	20
	6	11	11	30		6	1	1	21
	7	8	8	38		7	-	-	-
	8	15	15	53		8	-	-	-
	9	1	1	54		9	-	-	-
	10	20	20	74		10	1	1	22
	11	-	-	-		11	3	3	25
	12	-	-	-		12	1	1	26
	13	13	13	87		13	6	6	32
	14	-	-	-		14	1	1	33
	15	-	-	-		15	3	3	36
	16	-	-	-		16	1	1	37
	17	2	2	89		17	2	2	39
	18	-	-	-		18	1	1	40
	19	-	-	-		19	1	1	41
	20	-	-	-		20	59	59	100
	21	11	11	100		21	-	-	-
Total		100	100				100	100	

Statistical analysis. Descriptive analyses were performed using the PROC FREQ procedure of SAS software (SAS 2008). Agreement between tests was achieved by Kappa coefficient and McNemar test. Differences in results frequencies regarding bacteria type were determined by hierarchical models. Farm and animal effect were first assessed using the chi-square test. Variables individually associated with result test at a P-value ≤ 0.25 were selected to build a final generalized mixed model. Farm was included as an explanatory variable whereas animal did not enter the final models. To verify whether farm should be included as a fixed or random effect, individual generalized mixed models were constructed for each result test including farm as a random effect. A compound symmetry covariance structure was used to account for clustering of isolates from same farm. In cases of estimated V correlation values ≥0.20, models were building considering farm as a random effect. This applies to all beta-lactamases tests results (PCR, clover leaf test and Nitrocefin disk), whereas to penicillin susceptibility results, the model hierarchical structure assumed farm as a fixed effect (estimated V correlation value = 0.16). Each final model provided the best fit for the data, assessed using Akaike and Bayesian information criteria. Statistical significance was defined as $P \leq 0.05$. All statistical analyses were performed with the SAS 9.2 software.

RESULTS

PCR detection of *blaZ* gene was not performed in four isolates of CNS and two isolates of *Staphylococcus aureus*. Hence, these bacteria were excluded from pairwise analyses. Resistance to penicillin was detected in more *S. aureus* isolates (83/100) than CNS ones (32/100). Clover Leaf Test, Nitrocefin disks and PCR showed similar results, with 79.0%, 78.0% and 62.2% of positivity for *S. aureus*, and 29.0% 25.0% and 20.8%

for CNS for the three tests respectively. Several isolates from *S. aureus* and CNS displayed the beta-lactamase production phenotype detected by all three methods, whereas PCR results were negative (Table 3). Hence, agreement coefficients involving PCR test were lower when compared to other coefficients, particularly in *S. aureus* isolates (Table 2). For CNS, CLT/Nitrocefin and penicillin resistance/CLT had the two highest Kappa coefficients (0.84 and 0.79 respectively). The same was observed for *S. aureus* (CLT/Nitrocefin = 0.97; penicillin resistance/CLT = 0.80). Despite the high coefficient observed between penicillin resistance and Nitrocefin test for both species, McNemar's Test was significant for two tests, and in all three tests involving the PCR results for *S. aureus*.

According to hierarchical models applied, results of penicillin resistance (P=0.63), Nitrocefin (P=0.10), PCR (P=0.61) and CLT (P=0.18) were not dependent of bacteria type (CNS or *S. aureus*). Several *S. aureus* beta-lactamase producing isolates were from the same herd (herd 20, Table 1).

Table 2. Values of McNemar's P and kappa agreement coefficient with its respective lower (LB95%) and upper bounds (UB95%) of 95% confidence interval of different tests aiming beta-lactamases production/detection in *Staphylococcus aureus* and coagulase-negative *Staphylococcus* (CNS) isolates

	Test 1	Test 2	McNemar's P	Kappa	LB95%	UB95%
CNS	Penicillin	Clover Leaf	0.31	0.79	0.65	0.91
	Penicillin	Nitrocefin	0.01	0.78	0.64	0.91
	Penicillin	PCR	0.02	0.46	0.27	0.66
	Clover Leaf	Nitrocefin	0.1	0.84	0.73	0.96
	Clover Leaf	PCR	0.05	0.63	0.41	0.81
	Nitrocefin	PCR	0.56	0.63	0.44	0.82
S. aureus	Penicillin	Clover Leaf	0.1	0.8	0.65	0.95
	Penicillin	Nitrocefin	0.05	0.77	0.62	0.93
	Penicillin	PCR	0.001	0.46	0.29	0.63
	Clover Leaf	Nitrocefin	0.31	0.97	0.91	1
	Clover Leaf	PCR	0.001	0.54	0.37	0.71
	Nitrocefin	PCR	0.001	0.52	0.35	0.69

DISCUSSION

This study addressed an important topic: beta-lactamase production in *Staphylococcus aureus* and CNS, and comparison of diagnostic methods. Unsuccessful treatments are usually observed for mastitis caused by beta-lactamase producing isolates, particularly *S. aureus* (Sol et al. 2000). *S. aureus* is accepted as a major mastitis pathogen with laborious treatment, whereas CNS remains as a minor pathogen (Sampimon et al. 2009). In many countries, CNS have become the most common mastitis-causing agents (Pyorala & Taponen 2009), and is proving to be as pathogenic as *S. aureus*, at least concerning the presence of antimicrobial resistance genes. Studies regarding detection of resistance genes including *blaZ*, showed that none of the *S. aureus* possessed more than three genes, whereas 25% of CNS isolates harbored, at least, four genes encoding resistance to antibiotics (Podkowik et al. 2012).

In the present study, several *S. aureus* and CNS isolates showed beta-lactamase phenotype, in contrast to other studies (Johler et al. 2011). For all tests, *S. aureus* isolates showed beta-lactamase activity in a higher percentage than CNS. However, no significant difference was observed in all tests. Our hypothesis that more *S. aureus* isolates would produce beta-lactamases was not proved, despite the results observed in Table 3. We expected that due to a relative high rate of unsuccessful treatments (Leitner et al. 2003), more *S. aureus* isolates would present beta-lactamases enzymes. Our study showed that no difference was present between *S. aureus* and CNS isolates, and probably, beta--lactamase production is herd-dependent, although this was not studied. Recent studies showed that there is an association between antimicrobial use and antimicrobial resistance in dairy farms (Saini et al. 2012), even if there is very little evidence supporting an increase in antimicrobial resistance due to mastitis treatments (Erskine et al. 2002). Mastitis therapy differs between herds, which may

Table 3. Absolute and relative (%) frequencies of susceptibility to penicillin according to the disk diffusion method (S = susceptible; R = resistant), Clover Leaf Test, Nitrocefin Disk and PCR detection of the *blaZ* gene (N = negative; P = positive) in *Staphylococcus aureus* and coagulase--negative *Staphylococcus* (CNS) isolates enrolled in the study

Bacteria	Test	Result	Penicillin				Clover Leaf				Nitrocefin				PCR			
			S	%	R	%	N	%	P	%	N	%	P	%	N	%	P	%
CNS	Penicillin	S	-	-	-	-	65	65.0	3	3.0	67	67.0	1	1.0	61	63.5	5	5.2
		R	-	-	-	-	6	6.0	26	26.0	8	8.0	24	24.0	15	15.6	15	15.6
	Clover Leaf	N	65	65.0	6	6.0	-	-	-	-	70	70.0	1	1.0	66	68.8	3	3.1
		P	3	3.0	26	26.0	-	-	-	-	5	5.0	24	24.0	10	10.4	17	17.7
	Nitrocefin	N	67	67.0	8	8.0	70	70.0	5	5.0	-	-	-	-	69	72	5	5.2
		P	1	1.0	24	24.0	1	1.0	24	24.0	-	-	-	-	7	7.3	15	15.6
	PCR	N	61	63.5	15	15.6	66	68.8	10	10.4	69	71.9	7	7.3	-	-	-	-
		P	5	5.2	15	15.6	3	3.1	17	17.7	5	5.2	15	15.6	-	-	-	-
Bacteria	Test	Result	Penicillin				Clover Leaf				Nitrocefin				PCR			
			S	%	R	%	N	%	P	%	N	%	P	%	N	%	P	%
S. aureus	Penicillin	S	-	-	-	-	16	16.0	1	1.0	16	16.0	1	1.0	16	16.3	1	1.0
		R	-	-	-	-	5	5.0	78	78.0	6	6.0	77	77.0	21	21.4	60	61.2
	Clover Leaf	N	16	16.0	5	5.0	-	-	-	-	21	21.0	0	0.0	19	19.4	1	1.0
		P	1	1.0	78	78.0	-	-	-	-	1	1.0	78	78.0	18	18.4	60	61.2
	Nitrocefin	N	16	16.0	6	6.0	21	21.0	1	1.0	-	-	-	-	19	19.4	2	2.0
		P	1	1.0	77	77.0	0	0.0	78	78.0	-	-	-	-	18	18.4	59	60.2
	PCR	N	16	16.3	21	21.4	19	19.4	18	18.4	19	19.4	18	18.4	-	-	-	-
		P	1	1.0	60	61.2	1	1.0	60	61.2	2	2.0	59	60.2	-	-	-	-

contribute to different resistance profiles of similar bacteria. Herd 20 presented nearly 40% of all animals infected with *S. aureus* (data not shown), all harboring the *blaZ* gene. Unfortunately, we did not have access to information regarding mastitis therapy and prophylaxis from this herd, which could contribute to the understanding of *S. aureus* mastitis epidemiology.

In general, phenotypic tests showed a strong association measured by the kappa coefficient for both CNS and *S. aureus* (Table 2). The *blaZ* gene is widely spread among both *S. aureus* and CNS (Duran et al. 2012). In our study, we observed that several *S. aureus* and CNS isolates did not harbor the *blaZ* gene, and phenotypic tests showed beta-lactamase activity. This resulted in decreased kappa values, and in some cases, a significant statistical *P* value for McNemar's test (≤ 0.05; i.e., PCR x Penicillin resistance for CNS, PCR x CLT and PCR x Nitrocefin for *S. aureus*) showing that tests had a low agreement detecting beta-lactamase activity. Beta-lactamase phenotype could be result of expression of more than one gene, and there is more than one mechanism that grants staphylococci beta-lactam resistance other than the expression of *blaZ* gene (Malik et al. 2007). Moreover, the detection of a gene does not necessarily means it is expressed. Detection of RNAm could lead to more conclusions.

Two tests for both CNS and *S. aureus* involving resistance in vitro to penicillin had significant *P*-values, indicating that tests did not perform similarly detecting beta-lactamases. Several mechanisms could be involved in resistance to penicillin, and should be interpreted with caution to avoid misleading conclusions. In general, both Nitrocefin and CLT are specific to detect beta-lactamases production, and are more reliable tests for this purpose.

We choose to not assume PCR detection of the *blaZ* gene as a gold standard method for the same reasons we listed before, not calculating underestimated values of sensitivity and specificity for phenotypic tests, even with studies showing high sensitivity and specificity for Clover Leaf Test and Nitrocefin disks for detecting beta-lactamase production in staphylococci using the *blaZ* detection as the gold standard (Pitkala et al. 2007).

CONCLUSIONS

Staphylococcus aureus and CNS did not differ regarding production of beta-lactamases and detection of the *blaZ* gene.

All phenotypic tests performed similarly for both species, excluding in vitro resistance to penicillin.

Result of PCR detection of the *blaZ* gene had a low association with all phenotypic tests for both bacteria species.

There is a strong possibility that beta-lactamase production is herd-dependent.

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