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Comparison phenotypic and genotypic identification of *Staphylococcus* species isolated from bovine mastitis[1]

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ABSTRACT.- Guimarães F.F., Joaquim S.F., Manzi M.P., Silva R.C., Bruder-Nascimento A.C.M.O., Costa E.O. & Langoni H. 2016. **Comparison phenotypic and genotypic identification of *Staphylococcus* species isolated from bovine mastitis.** *Pesquisa Veterinária Brasileira 36(12):1160-1164*. Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Distrito de Rubião Jr s/n, Botucatu, SP 18618-970, Brazil. E-mail: hlangoni@fmvz.unesp.br

In addition to *Staphylococcus aureus* nowadays other coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS), earlier considered of minor importance, are now accepted as relevant pathogens for humans and animals. The involvement of these microorganisms in bovine mastitis etiology and the possibility their transmission through milk to humans justify the requirement of developing reliable methods for identification of the most frequent species among them. The purpose of this study was to compare the phenotypic techniques with the genotypic method carried out by sequencing of the *rpoB* gene in identification of several species of the genus *Staphylococcus* isolated from bovine mastitis. A total of 300 staphylococci isolates of bovine mastitis cases from several Brazilian dairy herds were studied by phenotypic and genotypic techniques, respectively: 150 CoPS and 150 CoNS strains. A total of 18 CoNS different species and 4 CoPS species were identified. Among the CoNS the following species were recognized: 48 (32%) *Staphylococcus warneri*, 22(15%) *S. epidermidis*, 20(13%) *S. hyicus*, 10(7%) *S. xylosus*, 7(5%) *S. haemolyticus*, 6(4%) *S. simulans*, 6(4%) *S. schleiferi* subsp *schleiferi*, 6(4%) *S. hominis*, 5(3%) *S. pasteuri*, 4(2.7%) *S. cohnii*, 3(2%) *S. saprophyticus* subsp. *saprophyticus* 3(2%) *S. chromogenes* 3(2%) *S. sciuri*, 2(1%) *S. saccharolyticus*, 2(1%) *S. lugdunensi*, 1(0,7%) *S. auricularis*, 1(70%) *S. saprophyticus* subsp. *bovis*, 1(0.7%) *S. capitis*. And among the 150 CoPS were identified respectively: 105 (70%) *S. aureus*, 21(14%), *S. hyicus*, 19(13%) *S. intermedius* e 5(3%) *S. schleiferi* subsp *coagulans*. Considering the 150 CoNS isolates, the identifications performed by phenotypic and genotypic tests presented 96.7% of concordance, kappa coefficient of agreement = 0.933, SE (standard error) of kappa=0.021 (95% confidence interval: 0.893 to 0.974), Pearson's correlation coefficient (r) = 0.9977, (confidence interval 95%: 0.9938 a 0.9992) and in relation to 150 CPS isolates it was detected an agreement of 98.7%, kappa = 0.960, SE of kappa = 0.016, (95% confidence interval: 0.929 to 0.992) Pearson's correlation coefficient (r) = 0.9994 (95% confidence interval: 0.9681 to 1.0000). The verified agreement strength between the identification methods can be considered as excellent. These results assure that according to laboratory resources any of them will be suitable to perform the staphylococci identification.

INDEX TERMS: Phenotype, genotype, *Staphylococcus* spp., mastitis, sequencing, *rpoB* gene, biochemical tests, coagulase positive *Staphylococcus*, coagulase negative *Staphylococcus.*

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RESUMO.- [**Comparação da identificação fenotípica e genotípica de espécies do gênero *Staphylococcus* isoladas de mastite bovina.**] Além de *Staphylococcus aureus* atualmente outros estafilococos coagulase positiva (SCP) e estafilococos coagulase-negativos (SCN), anteriormente considerados de menor relevância, são reconhecidos como importantes patógenos para humanos e animais. O envolvimento desses micro-organismos na etiologia da mastite bovina e a possibilidade da sua transmissão através do leite aos humanos justifica a utilização de métodos confiáveis para a identificação das espécies mais frequentes. O objetivo deste estudo foi comparar as técnicas fenotípicas com o método genotípico realizada por sequenciamento do gene *rpoB* na identificação de espécies do gênero *Staphylococcus* spp. isolados de mastite bovina. Um total de 300 estafilococos isolados de casos de mastite bovina em diferentes rebanhos leiteiros brasileiros foram estudados por técnicas fenotípicas e genotípicas, respectivamente: 150 linhagens de SCP e 150 linhagens de SCN. Foram identificados um total de 18 espécies de SCN e 4 espécies SCP. Entre os SCN as seguintes espécies identificadas: 48 (32%) *Staphylococcus warneri*, 22 (15%) *S. epidermidis*, 20 (13%) *S. hyicus*, 10 (7%) *S. xylosus*, 7 (5%) *S. haemolyticus*, 6 (4%) *S. simulans*, 6 (4%) *S. schleiferi* subsp *schleiferi*, 6 (4%) *S. hominis*, 5 (3%) *S. pasteuri*, 4 (2,7%) *S. cohnii*, 3 (2%) *S. saprophyticus* subsp. *saprophyticus,* 3 (2%) *S. chromogenes,* 3 (2%) *S. sciuri*, 2 (1%) *S. saccharolyticus*, 2 (1%) *S. lugdunensi*, 1 (0,7%) *S. auricularis*, 1 (70 %) *S. saprophyticus* subsp. *bovis*, 1 (0,7%) *S. capitis*. E entre as 150 SCP foram identificados, 105 (70%) *S. aureus*, 21 (14%), *S. hyicus*, 19 (13*%) S. intermedius* e 5 (3%) *S. schleiferi* subsp *coagulans*. Considerando-se os 150 SCN isolados, as identificações realizadas por testes fenotípicos e genotípicos apresentaram 96,7% de concordância, coeficiente de concordância kappa = 0,933, SE (erro padrão) de kappa = 0,021 (95% intervalo de confiança: 0,893-0,974), coeficiente de correlação de Pearson (r) = 0,9977, (intervalo de confiança de 95%: 0,9938 a 0,9992) e em relação a 150 SCP isolados foi observado uma concordância de 98,7%, kappa = 0,960, sE de kappa = 0,016, (95% de intervalo de confiança: 0,929 a 0,992) coeficiente de correlação de Pearson (r) = 0,9994 (95% intervalo de confiança: 0,9681-1,0000). A correlação entre os métodos de identificação pode ser considerada como excelente. Esses resultados demonstraram que de acordo com os recursos disponíveis no laboratório, poderia ser utilizada qualquer uma das metodologias.

TERMOS DE INDEXAÇÃO: Fenotipo, genótipo, *Staphylococcus spp.,* mastite, sequenciamento, gene *rpoB*, testes bioquímicos, *Staphylococcus* coagulase positiva, *Staphylococcus* coagulase negativa.

INTRODUCTION

Knowledge of the phenotypic, genotypic characteristics of a microorganism is imperative for the detection of the pathogenicity and/or toxins that are detrimental to the health of plants, animals, humans and the environment. As well as, the accurate identification of the microorganism is a fundamental component for the establishment of safety measures (Hennekinne et al. 2010).

Dairy cow mastitis is the most important disease in the dairy industry worldwide. This disorder causes relevant economical losses, due to reduced milk production and poor milk quality, increasing the cost of treatment, and the premature disposal of cows (McDougall et al. 2009). One of the main agents of bovine contagious mastitis is *Staphylococcus aureus* (Capurro et al. 2010). Nowadays, the intramammary infections caused by other species of the genus *Staphylococcus* become a serious problem for dairy herds (Ergün et al. 2009, Pyorala & Taponen 2009, Unal & Yildirim 2010, Kunz et al. 2011, Guimarães et al. 2013, Langoni et al. 2015).

Furthermore the emergence of other species besides *Staphylococcus aureus* as human pathogens, as well as, reservoirs of antimicrobial resistance and other virulence factor, increases the necessity in the developing of reliable methods for the most frequent species identification, to establish the pathogen-host relationship, as well, development of epidemiological approaches (Silva et al. 2013, Silva et al. 2014, Rall et al. 2014, Robles et al. 2014).

Coagulase-negative *Staphylococcus* (CoNS) have become increasingly important in human and veterinary medicine, therefore it is mandatory accurately identify the isolates to species level in order to define the clinical significance of these bacteria, to carry out a proper epidemiological surveillance, and to manage patients infected with CoNS in case of relapse (Poyart et al. 2001).

The genus *Staphylococcus* currently includes about 50 species e subspecies (Hennekinne et al. 2010). The identification of staphylococci species other than *S. aureus* is not regularly made in clinical laboratories of microbiology, because it is expensive, even with automation, and is time-consuming when carried out manually (Edwards et al. 2001).

Therefore, the purpose of this study was to compare phenotypic techniques with genotypic method in the identification of several different species of genus *Staphylococcus* isolated from bovine mastitis cases.

MATERIALS AND METHODS

A total of 300 staphylococci isolates of bovine mastitis cases collected from several Brazilian dairy herds were studied, respectively: 150 CoPS and 150 CoNS strains.

The methodology and interpretation criteria used for diagnosis of clinical and subclinical mastitis were based on examination of animals before each milking by the strip cup and the California Mastitis Test - CMT (Schalm & Noorlander 1957). Milk samples were collected in sterile tubes aseptically, and they were transported to the laboratory under refrigeration (4-8°C) in cool boxes with ice packs.

Bacterial isolates. The samples were plated on blood agar (5%) (Oxoid) and incubated under aerobic conditions at 37°C, and readings were performed after 24, 48 and 72 hours of incubation. Identification of staphylococci was based on colony morphology, Gram staining, catalase, coagulase and DNase activities (Koneman et al. 2008).

Staphylococcus spp. were differentiated from *Micrococcus* spp. based on oxidation and fermentation of glucose, resistance to bacitracin (0.04 U), and susceptibility to furazolidone (100μg) (Baker 1984).

Identification of *Staphylococcus* spp. was carried out according to the criteria suggested by Kloos & Schleifer (1975) modified by Cunha et al. (2004). Series of biochemical tests were performed such as sugar fermentation (xylose, arabinose, sucrose, trehalose, maltose, mannitol, lactose, xylitol, ribose, fructose, and mannose), production of hemolysin, nitrate reduction, presence of urease and ornithine decarboxylase, and resistance to novobiocin.

To confirm the identification of individual species, the following international reference CoNS strains were used as controls: *Staphylococcus auricularis* (ATCC 33753), *S. capitis* subsp. *capitis* (ATCC 27843), *S. capitis* subsp. *urealyticus* (ATCC 49325) *S. caprae* (ATCC 35538), *S. cohnii* (ATCC49330), *S. cohnii* subsp. *cohnii* (ATCC 29974), *S. epidermidis* (ATCC12228 e 35983), *S. haemolyticus* (ATCC 29970), *S. hominis* (ATCC 27844), *S. hominis* subsp. *novobiosepticus* (ATCC 700237), *S. lentus* (ATCC 700403), *S. lugdunensis* (ATCC 700328), *S. saprophyticus* (ATCC 15305), *S. schleiferi* subsp. *schleiferi* (ATCC 43808), *S. sciuri* subsp. *sciuri* (ATCC 29062) *S. simulans* (ATCC 27851), *S. warneri* (ATCC 10209), *S. xylosus* (ATCC 29979) and *S. aureus* (ATCC 33591 and ATCC 25923).

Gene *rpoB* detection. In all the isolates CoNS and CoPS were performed gene *rpoB* detection, the gene encoding the beta subunit of RNA polymerase, by sequencing amplified region, using the *primers* (Mellmann et al. 2006) the following parameters was used: 899 pb, rpob1418for 5'-CAATTCATGGACCAAGC-3' and rpob3554rev 5'-CCGTCCAAGTCATGAAAC-3', in 25 µL there was 10 pmol of each *primer*, 2,5 U de Taq DNA polimerase, 200 µM de desoxirribonucleotides thrifosfate, 20 mM de Tris-HCL (pH 8,4), 0,75mM de MgCl2 e 3 µL de DNA. The amplification was performed by Mastercycler gradient® (Eppendorf) termal cycler during 5 minutes at 94 ºC, followed by 35-seconds cycles of denaturation at 94 ºC during 45 minutes, and the primers annealing one minute at 52oC and, one minute and thirty seconds at 72 °C. The program was completed with an additional extension of 10 minutes at 72 °C. The efficiency of the amplifications was monitored by electrophoresis of the reaction in 2% UltrapureTM agarose gel prepared in o.5 Tris-Borate-EDTA(TBE) buffer. A marker with molecular weight of 100bp was used as a standard. DNA was stained by Sybr Safe® and later photographed under ultraviolet illumination**.**

Sequencing. The *rpoB* regions amplicons of CoNS and CoPS were first submitted to purification by GFX PCR kit and Gel Band Purification (GE Healthcare®), subsequently the sequencing reactions were carried out in DNA ABI (Applied Biosystems) Prism model 377. The *rpoB* sequences were aligned by using the multisequence alignment Mega 5.2 program analyzed in GenBank using Blat tool in order of compare the sequences.

Statistical analysis. Statistical analyzes were performed with GRAPHPAD INSTAT software (Statistical Analysis Systems for personal computers, 1990-1993), using the test pearson's correlation coefficient (r) and it were analyzed the level of agreement, obtaining the kappa value (<0 = no agreement; 0-0.2 = slight agreement; 0.21-0.4 = fair agreement; 0.41-0.6 = moderate agreement; 0.61-0.8 = substantial agreement; 0.8-1 = almost perfect agreement). The K linear weighting test was calculated directly in the following site http://www.graphpad.com/quickcalcs/kappa.

RESULTS

Among the 150 CoNS isolates from bovine mastitis cases eighteen different species were identified respectively: *Staphylococcus warneri*, *S. epidermidis*, *S. hyicus*, *S. xylosus*, *S. haemolyticus*, *S. simulans*, *S. schleiferi* subsp *schleiferi*, *S. hominis*, *S. pasteuri*, *S. cohnii*, *S. saprophyticus* subsp. *saprophyticus*, *S. chromogenes*, *S. sciuri*, *S. saccharolyticus*, *S. lugdunensi*, *S. auricularis*, *S. saprophyticus* subsp. *bovis*, *S. capitis*. The results of phenotypic and genotypic identifications were presented on Table 1, the methods showed a 96.7% of concordance, and it was verified Pearson's correlation coefficient (r) = 0.9977 (95% confidence interval: 0.9938 to 0.9992) and, Kappa = 0.933, SE of kappa = 0.021 (95% confidence interval: 0.893 to 0.974).

And four different species were identified among the 150 CoPS isolates: *S. aureus*, *S. hyicus*, *S. intermedius* and *S. schleiferi* subsp *coagulans*. The results of phenotypic and genotypic identifications were presented on Table 2, the methods showed 98.7% of concordance, Pearson's correlation coefficient (r) = 0.9994 (95% confidence interval: 0.9681 to 1.0000) and, Kappa = 0.960, SE of Kappa = 0.016 (95% confidence interval: 0.929 to 0.992).

DISCUSSION

In the last years, CoNS has attracted larger attention due to her pathogenicity and involvement in human and animal diseases. The importance of identifying the species of CoNS in the clinical laboratories, for the determination of their physiopathological and epidemiological characteristics has been increasing. However, it is not an easy task, since phe-

Table 1. Phenotypic and genotypic identification of the 150 CoNS of bovine mastitis cases isolates from Brazillian dairy herds. 2016

Species	Phenotypic		Genotypic	
	Nº	%	Nº	%
Staphylococcus auricularis	1	0.7	1	0.7
S. capitis	1	0.7	1	0.7
S. chromogenes	1	0.7	3	2.0
S. cohnii subsp cohnii	4	2.7	4	2.7
S. epidermidis	22	14.7	22	14.7
S. haemolyticus	7	4.70	7	4.7
S. hominis	6	4.0	6	4.0
S. hyicus	22	14.7	20	13.3
S. lugdunensis	2	1.3	2	1.3
S. pasteuri	5	3.3	5	3.3
S. saccharolyticus	2	1.3	2	1.3
S. saprophyticus subsp bovis	1	0.7	1	0.7
S. saprophyticus subsp. saprophyticus	3	2.0	3	2.0
S. schleiferi subsp schleiferi	5	3.3	6	4.0
S. sciuri subsp sciuri	3	2.0	3	2.0
S. simulans	4	3.0	6	4.0
S. warneri	50	33.0	48	32.0
S. xylosus	11	7.3	10	6.7
Total	150		150	

Concordance = 96.7%; Kappa = 0.933; SE of kappa = 0.021(95%; Confidence interval = 0.893 to 0.974)

Table 2. Phenotypic and genotypic identification of the 150 CoPS isolates of bovine mastitis cases isolates from Brazilian dairy herds. 2016

Species	Phenotypic		Genotypic	
	Nº	%	Nº	%
Staphylococcus hyicus	21	14.0	19	12.7
S. intermedius	19	12.7	21	14.0
S. schleiferi subsp coagulans	5	3.3	5	3.3
S. aureus	105	70.0	105	70.0
Total	150		150	

Concordance = 98.01%; Kappa = 0.960; SE of kappa = 0.016 (95%; Confidence interval = 0.929 to 0.992)

notypic tests can present similar results, hindering the obtaining of a result, as well as a great expense of time in the identification of the species (Bannerman 2003, De Paulis et al. 2003).

On the other hand, PCR sequencing of the *rpoB* gene effectively identify staphylococcal species. In contrast to the probe hybridization technique and the RFLP approach, sequencing enables any isolate to be characterized, including new species by their phylogenetic relationships (Drancourt & Raoult 2002). And these authors concluded that the partial sequencing of the *rpoB* gene as a suitable new tool for the accurate identification of Staphylococcus isolates.

Concordance analysis is needed to establish the validity of a new diagnostic measuring or rating technique or to demonstrate the near-equivalence of multiple measuring or rating techniques. In the present study the comparison of the results obtained using the biochemical phenotypic method and sequencing of the *rpoB* gene for isolates identification showed a high concordance, 96.7% for the 150 CNS isolates and the kappa equal 0.933. As well as, in respect to the 150 CPS isolates it was observed a concordance of 98.1% and kappa equal 0.960. According to Landis & Koch (1977) these obtained values of kappa were indicative of high agreement between the methods. Landis & Koch (1977) have characterized different ranges of values for kappa with respect to the degree of agreement, they suggest for most purposes, values greater than 0.75 or so may be taken to represent excellent agreement beyond chance, values below 0.40 or so may be taken to represent poor agreement beyond chance, and values between 0.40 and 0.75 may be taken to represent fair to good agreement beyond chance.

When a new assay or instrument was developed it is of interest to evaluate whether the new assay can reproduce the results based on a traditional the gold-standard assay (Bauer & Kennedy 1981), such validation processes are often evaluated using the Pearson correlation coefficient. The obtained identification results showed a Pearson's correlation coefficient (r) = 0.9977, (confidence interval 95%: 0.9938 to 0.9992) to CoNS and, Pearson correlation coefficient (r) = 0.9994, (95% confidence interval: 0.9681 to 1.0000) to CoPS.

Dancey & Reidy (2006) referred that is considered a strong correlation when the value of Pearson correlation coefficient was = 0.70 to 1, consequently the obtained results indicated a high correlation between the two methods.

From the 300 staphylococci isolates of bovine mastitis cases twenty-two different species were identified through the phenotypic technique proposed by Kloos & Schleifer (1975), modified by Cunha et al. (2004) and genotypic technique carried out by sequencing of the *rpoB* gene.

It is important to point out the great diversity of CoNS species in the etiology of bovine mastitis and that some of them were also relevant in human nosocomial cases, for example the most frequent species identified in the present study *S. warneri*, *S. epidermidis* and *S. hyicus*, among the CoNS (Table 1), *S. aureus* and *S. intermedius* among the CoPS (Table 2).

In the current study the highest frequencies observed were *S. aureus* 70% among the 150 CoPS, *S. warneri* 32% and *S. epidermidis* 15% among the 150 CoNS isolates of bovine mastitis cases. The significance of *S. aureus* was well recognized not only to to animal health as well to human health. It is also imperative to point out the relevance of those other species to public health in view of the fact that *S. epidermidis* frequency among human isolates ranged from 43% to 92%, depending on the geographical region where the study was conducted (Ieven et al. 1995, Couto et al. 2001, Vuong & Otto 2002, De Paulis et al. 2003, Spanu et al. 2003, Cunha et al. 2004, Caierão et al. 2006). Among hospitalized Brazilian patients Pereira (2014) studying 300 CoNS isolated by blood cultures from verified that *S. epidermidis* was the most frequent identified specie (74.3%) and followed by *S. haemolyticus* 27 (9.0%), *S. hominis* 22 (7.3%), *S. warneri* 14 (4.7%), *S. lugdunensis* 9 (3.0%) e *S. capitis* 5 (1.7%). Kamath et al. (1992) reported twenty-seven episodes of bacteremia nosocomially acquired caused by *S. warneri*. Literature review of reported infections with *S. warneri* include catheter related or unrelated bacteremias with or without immunosuppression, endocarditis (Cimiotti et al. 2007), neonatal infections (Buttery et al. 1997). Considering the animal health, besides the relevance of *S. epidermidis and S. warneri* on bovine mastitis etiology (Siqueira 2011, Guimarães et al. 2013), *S. warneri* was accountable for bovine abortion (Barigye et al. 2007) and canine meningoencephalitis (Espino et al. 2006).

CONCLUSION

Microbiology laboratories require rapid, sensitive and specific techniques to perform identification tests. In the last decades, new molecular methods have been developed and introduced in clinical laboratories of microbiology to improve the credibility of these tests. However, for most clinical laboratories these techniques are still very expensive and phenotypic identification is the most common method used. The present study showed high concordance in the comparison of these methodologies these results assure that according to laboratory resources any of them will be suitable to perform the staphylococci identification.

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