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BIOCHEMICAL DIFFERENTIATION AMONG *S. aureus, S. Intermedius* AND *S. hyicus* ISOLATED FROM BOVINES WITH SUBCLINICAL MASTITIS

(Diferenciação bioquímica entre S. aureus, S. Intermedius e S. hyicus isolados em bovinos com mastite subclinica)

GANDRA, E.A.[1]; SILVA, J.A.[3]; MACEDO, M.R.P.[2]; ARAÚJO, M.R.[1]; MATA, M.M.[2]; SILVA, W.P.[3]

[1]Doutorado do Programa de Pós-Graduação em Ciência e Tecnologia Agroindústria;
[2]Bolsista de Iniciação Científica;
[3]Docente do Departamento de Ciência e Tecnologia Agroindustrial, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas.

RESUMO – *Staphylococcus aureus* é, entre as espécies de estafilococos, a mais relacionada a infecções em animais e humanos, bem como a doenças de origem alimentar. Entretanto, *S. intermedius* e *S. hyicus*, duas espécies com características morfológicas muito similares a *S. aureus*, também podem causar doenças, bem como produzir enterotoxinas em alimentos, tornando importante a diferenciação entre essas três espécies de estafilococos. O objetivo deste trabalho foi avaliar a eficiência de oito técnicas bioquímicas com relação à sua capacidade para distinguir entre *S. aureus, S. intermedius* e *S. hyicus*. Para isso, 65 cepas, previamente identificadas em nível de espécie através de técnicas moleculares, foram submetidas aos testes de produção de pigmentos carotenóides, atividade hemolítica em ágar sangue, produção de β- galactosidase, produção de acetoína, atividade lipolítica em polisorbato, fermentação aeróbica da maltose, fermentação anaeróbica do manitol e crescimento em ágar Baird-Paker e ágar P suplementados com acriflavina. Verificou-se que os testes de sensibilidade a acriflavina e de produção de β- galactosidase apresentaram bom poder discriminatório, demonstrando serem os melhores testes bioquímicos para a diferenciação entre essas três espécies de estafilococos.

Palavras-chave: *S. aureus*, *S. intermedius*, *S. hyicus,* diferenciação bioquímica.

ABSTRACT – Among staphylococcus species, *Staphylococcus aureus* is the one mostly related to animal and human infections, as well as food diseases. However, *S. intermedius* and *S. hyicus*, two species with very similar morphological characteristics to *S. aureus*, can also cause diseases as well as produce enterotoxins in food, what makes important the differentiation of these three species. The aim of this work was to study the efficiency of eight biochemical techniques regarding their capacity to distinguish among *S. aureus*, *S. intermedius* and *S. hyicus*. For that purpose, sixty five strains, previously identified at species level through molecular techniques, were submitted to the tests of carotenoid pigments production, hemolytic activity in blood agar, β- galactosidase production, acetoin production, lipolytic activity in polysorbate, maltose aerobic fermentation, manitol anaerobic fermentation and growth in Baird-Parker and P agar supplemented with acriflavine. It was verified that tests of sensitivity to acriflavine and β-galactosidase production display good distinguishing properties, constituting as a whole, the best biochemical tests for the identification of these three staphylococcus species.

Key-words: *S. aureus*, *S. intermedius*, *S. hyicus,* biochemical differentiation.

Autor para correspondência: SILVA, W.P. – Fone: 53-2757378, Fax: 53-2759031 – e-mail: silvawp@ufpel.tche.br.

Introduction

Staphylococcus are bacteria from the *Micrococcaceae* family, which include agents of several bovine infections, including mastitis. Besides, some species can produce enterotoxins in foods, which when ingested, can cause food poisoning in humans. Nowadays, 32 *staphylococcus* species are described, from which, five are denominated coagulase positive *staphylococcus* (*S. aureus, S. hyicus, S. intermedius, S. delphini* and *S. schleferii, subsp. coagulans*), because they produce an extracellular enzyme which is coagulase (JAY, 1992; KONEMAM *et al*., 1997; KLOOS and BANNERMAN, 1999). From these, three are pathogenic for both, bovines and humans (*S. aureus, S. hyicus* e *S. intermedius).* Besides being primary pathogens of the mammary gland of bovines causing contagious mastitis (MATTHEWS *et al*. 1990; SILVA *et al*., 2000), they are capable of producing enterotoxins, having already been associated to food poisoning outbreaks (JAY, 1992; KLOOS and BANNERMAN, 1999, ADESIYUN *et al*., 1984, BECKER *et al*, 2001, CENGI-GOGA *et al*, 2003)

These three species present very similar morphologic characteristics when grown in selective and differential media (BANDEIRA, 2001, LANCETTE and BENETT, 2001). The isolation, followed by coagulase and thermonuclease production tests (methodology normally used in the laboratory), may not be enough neither to identify and differentiate the three species, nor to guarantee the absence of these microorganisms in food, because apart from the similarities previously described, the three microorganisms produce coagulase and thermo-nuclease enzymes.

Studies trying to determine the minimal number of biochemical tests which could be used to identify *S. aureus*, *S. intermedius* and *S. hyicus* have already been published (CAPURRO *et al*., 1999; ROBERSON *et al*., 1992), however there is not an agreement regarding which would be the best tests for identification and differentiation of these three species. This work was carried out with the aim of evaluating the efficiency of eight biochemical techniques usually used in microbiology laboratories, regarding their capacity to distinguish among *S. aureus*, *S. intermedius* and *S. hyicus*.

Material and methods

Bacterial strains: Sixty five strains isolated from udder skin and from milk of cows with subclinical mastitis were used. They had previously been characterized as Coagulase Positive Staphylococcus (CPS) through Gram differential staining test and catalase, coagulase and thermo-nuclease production. Gram +, catalase, thermo-nuclease and coagulase producers strains were identified at species level by species-specific PCR (data not shown), as *S. aureus* (55 strains), *S. intermedius* (4 strains) e *S. hyicus* (6 strains).

A standard strain of *S. aureus* (ATCC 10832) and *S. intermedius* and *S. hyicus* strains isolated from clinical samples, kindly provided by the Veterinary Faculty of Federal University of Pelotas, Pelotas, Brazil, were used as controls.

Biochemical identification: The biochemical tests were interpreted according to criteria proposed by ROBERSON *et al*. (1992), BASCOMB and MANAFI (1998), CAPURRO *et al*. (1999), KLOOS and BANNERMAN (1999) and OLIVEIRA (2000), as shown in TABLE 1.

For evaluation of the carotenoid pigments production, the CPS strains were inoculated in brain and heart infusion agar (BHA, Oxoid) and incubated at 37°C for 5 days, according to GILL *et al*. (1994).

The hemolytic activity was determined in petri dishes with 5% sheep blood agar following the recommendations of KONEMAM *et al*. (1997). After inoculation, the plates were incubated at 37°C for 24 to 48 hours.

The maltose aerobic fermentation test was made using petri dishes containing Bromocresol purple agar (Bromocresol purple broth, Oxoid and Agar-agar, Merck), with a final concentration of 1% maltose (Vetec). The plates were streaked with CPS cultures and incubated at 37°C for up to 72 hours, following the protocol proposed by KONEMAM *et al*. (1997). The mannitol anaerobic fermentation was determined according to the procedures described by ROBERSON *et al*. (1992). Tubes

with 4 mL of Bromocresol purpura broth (Oxoid), with final concentration of 1% mannitol (Vetec), were prepared. The tubes were inoculated, mineral oil (0.5 mL) was added and incubated at 37°C, for up to 5 days. The acetoin production was evaluated as described by CHAPIN and MURRAY (1999), in test tubes with 1.0 mL of Methyl Red-Voges Proskauer broth (MR-VP, Oxoid) inoculated and submitted to 48 hours incubation at 37°C. The reading was carried out after this period, with the addition of 0.6 mL of α-naftol 5% (p/v) in absolute alcohol and 0.2 mL of KOH at 40%.

The lipolytic activity in polysorbate was determined from a fresh culture (24 h at 37°C) in brain heart infusion broth (BHI, Oxoid), from which, plates containing Tween 80 agar streaked and incubated at 37°C for 24 to 48 hours, according to the protocol proposed by CHAPIN and MURRAY (1999).

Production of β-galactosidase was determined according to ROBERSON *et al*. (1992), using as a substrate the ortho-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma). A loop full of a fresh culture (24 hours) grown in Triple Sugar and Iron agar (TSI, Merck), was inoculated in 0.5 mL of ONPG broth (CHAPIN and MURRAY, 1999) and incubated in water bath at 37°C for one hour.

In order to verify the sensitivity to acriflavine, P agar (formulated according to CHAPIN and MURRAY, 1999) and Baird-Parker agar (Oxoid), with and without supplementation with 7 μg.mL^{-1} of acriflavine (Sigma), were streaked with a fresh culture (24 hours at 37°C in BHI broth) and incubated at 37°C for 24 and 48 hours respectively, as described by ROBERSON at al. (1992).

Results

It has been found that fifty five strains of *S. aureus* grew in Baird-Parker agar (ABP) and P agar (AP) supplemented with acriflavine and did not produce β-galactosidase, however, they presented variable results in the other biochemical tests used: 13 strains produced carotenoid pigments, 36 fermented maltose aerobically, 47 fermented mannitol anaerobically, 46 produced acetoin and 40 presented hemolytic activity in blood agar.

Among the six strains of *S. hyicus,* four presented negative result in all biochemical tests performed, whereas two grew in Tween 80 agar

All four strains of *S. intermedius* produced β-galactosidase and gave negative results in the anaerobic mannitol fermentation, growth in ABP and AP supplemented with acriflavine and in the test of pigments production. Like *S. aureus,* these strains presented variability in their biochemical characteristics: one fermented maltose, one presented hemolytic activity in Blood agar and two produced acetoin.

TABLE 2 shows the percentage of strains of *S. aureus*, *S. intermedius* and *S. hyicus* with positive results in the biochemical tests used.

TABLE 1 – BIOCHEMICAL DIFFERENTIATION AMONG *S. aureus, S. intermedius* AND *S. Hyicus.*

Species	Pig[a]	Hem[b]	β-gal[c]	Acet[d]	Mal[e]	Man[f]	Pol[g]	Acri[h]
S. aureus	+	+	-	+	+	+	-	+
S. intermedius	-	d	+	-	(+)	-	-	-
S. hyicus	-	-	-	-	-	-	+	-

Symbols: +, ≥ 90% of the species or strains are positive; -, ≥ 90% of the species or strains are negative; d, 11 to a 89% of the strains are positive; ( ), slow reaction.
Source: Adapted from ROBERSON *et al*. (1992), CAPURRO *et al*. (1999), KLOOS and BANNERMAN (1999) and OLIVEIRA (2000).
[a]Carotenoid pigment production; [b]Hemolytic activity in 5% Sheep Blood Agar; [c]β-galactosidase production; [d]Acetoin production; [e] Maltose aerobic fermentation;
[f] Mannitol anaerobic fermentation; [g] Lipolytic activity in Polysorbate (tween 80);
[h]Growth in modified Baird-Parker agar, supplemented with acriflavine (7 μg.mL^{-1});
[i] Growth in P modified Agar, supplemented with acriflavine (7 μg.mL^{-1}).

TABLE 2 – PERCENTAGE OF STRAINS OF *S. aureus*, *S. intermedius* AND *S. HYICUS* WITH POSITIVE RESULT IN 8 BIOCHEMICAL TESTS OF IDENTIFICATION.

Specie	Pig[a]	Hem[b]	β-gal[c]	Acet[d]	Mal[e]	Man[f]	Pol[g]	ABPm[h]	APm[i]
S. aureus	23.6	72.7	0.0	83.6	65.4	85.4	0.0	100	100
S. intermedius	0.0	25.0	100.0	50.0	25.0	0.0	0.0	0.0	0.0
S. hyicus	0.0	0.0	0.0	0.0	0.0	0.0	33.3	0.0	0.0

For symbols see p. 77.

Discussion

It was observed that 65% of the *S. aureus* strains and 25% of *S. intermedius* strains fermented maltose anaerobically and that no strain of *S. hyicus* was capable of fermenting this carbohydrate. The low percentage of strains of *S. intermedius* which used maltose can be explained by the low fermentative capacity of this species regarding this carbohydrate as reported by ROBERSON *et al*. (1992). In relation to *S. hyicus,* it was observed that neither of the strains fermented maltose, which agrees with JABLONSKI and BOHACH (1997), who described this species as a non fermentor of this carbohydrate.

By analyzing the hemolytic activity in Sheep Blood agar, it was verified that 72.7% of *S. aureus* and 25% of *S. intermedius* presented positive reactions, but it was not found hemolytic activity with *S. hyicus*. These results agree with ROBERSON *et al*. (1996), who reported that *S. aureus* and *S. intermedius* are capable of producing hemolysis in Blood agar. However, it must be pointed out that non hemolytic strains of both species can be found.

In the present work, 85.4% of *S. aureus* strains were able to ferment mannitol anaerobically, whereas no strain of *S. hyicus* or *S. intermedius* fermented this carbohydrate. ROBERSON *et al*. (1992) working with 80 strains of each of these three staphylococcus species, from different origins and CAPURRO *et al*. (1999), using 414 strains of ECP isolated from milk from cows with subclinical mastitis, found higher percentages of *S. aureus* strains with this characteristic: 99% and 100%, respectively. Both publications reported that no strain of *S. hyicus* or *S. intermedius* was able to ferment mannitol under anaerobiosis, agreeing with the results of this study.

Forty six strains (83.6%) of *S. aureus* produced acetoin. ROBERSON *et al*. (1992) and CAPURRO *et al*. (1992), observed higher percentages: 94% and 98% respectively. Two strains of *S. intermedius* produced acetoin, which was also observed by CAPURRO *et al*. (1999), who found two out of eight strains (25%) with positive result in this test. Regarding *S. hyicus,* no strain produced this compound, agreeing with the works of ROBERSON *et al*. (1992), CAPURRO *et al*. (1999) and KLOOS and BANNERMAN (1999), who reported that this species do not produce acetoin.

The lipolytic activity in Tween 80 agar was suggested by OLIVEIRA (2000) as the best test to differentiate *S. hyicus* (lipolytic in this medium) from *S. aureus* (not lipolytic in the same medium). In this work, none of the staphylococcus identified as *S. aureus* or as *S. intermedius* presented positive reaction in this test. However, only two *S. hyicus* strains (33%) presented lipolytic activity. Similar results were reported by KINBEGE *et al*. (1983) who found six strains (41.1%) not lipolytic in Tween 80 agar, from a total of 13 strains of *S. hyicus* isolated from chicken.

The percentage of strains of *S. aureus* that produced carotenoid pigments in BHA (23.6%) is below the one found by GIL *et al*. (1994), who, working with 116 strains of *S. aureus* isolated from milk, dairy products, beef, meat products and from equipment surfaces and on the hands of the people who handle food, found that 87% of the strains produced these pigments. It must be said that the low percentage found in this study, may have been influenced by factors such as culture medium, temperature and incubation time, according to KLOOS and BANNERMAN (1999).

The β-galactosidase production test (ONPG test) presented high discriminatory power, considering that 100% of the strains of *S. aureus* and *S. hyicus,* presented negative result and that 100% of the *S. intermedius* strains produced this enzyme, fact also observed by ROBERSON *et al*. (1992) and CAPURRO *et al*. (1999). These results demonstrated a great usefulness of this test to differentiate *S. intermedius* from other coagulase positive species. Besides, β-galactosidase production test was, among the tests evaluated, the one that allowed the reading of the results in less time, with an incubation period of only one hour.

HARMON *et al*. (1991) verified that 155 (99.3%) of 156 *S. aureus* strains isolated from bovine milk grew in P agar supplemented with 7 μg of acriflavine per milliliter, whereas only one (10%) out of 10 *S. intermedius* strains isolated from dogs, was able to grow in this medium, however the growth was weak. ROBERSON *et al*. (1992) and CAPURRO *et al*. (1999) verified that 100% of the strains of *S. aureus* grew in P and ABP agar supplemented with acriflavine (7 $\mu g.mL^{-1}$) and that none of *S. intermedius* or *S. hyicus* isolates was able to grow in these media. In agreement with these results, in this study, all the 55 strains of *S. aureus* grew in AP and ABP supplemented with acriflavine (7 $\mu g.mL^{-1}$), whereas no strain from *S. intermedius* or *S. hyicus* grew in these media.

It was verified that the tests of sensitivity to acriflavin and β-galactosidase production showed higher discriminatory power in relation to the other biochemical tests, considering that there was no variability of results among the strains of each of the three species of ECP, which allowed identified with higher precision. The results suggest that the joint use of these two tests allow the differentiation among *S. aureus*, *S. intermedius* and *S. hyicus*, in accordance with ROBERSON *et al.* (1992) and CAPURRO *et al.* (1999), who also report that these tests are the most precise for the differentiation of these three microbial species.

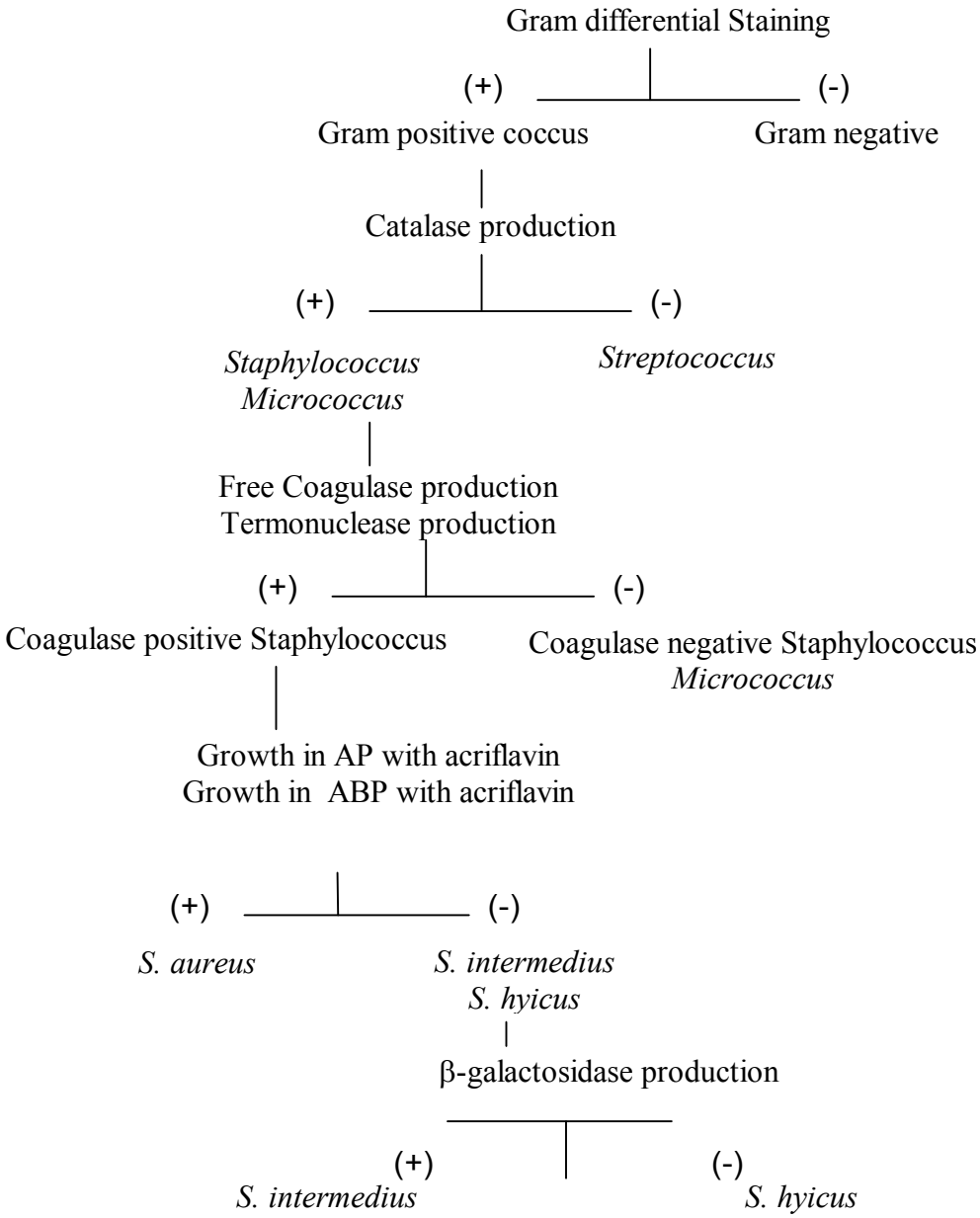
The mannitol anaerobic fermentation and acetoin production tests also presented satisfactory discriminatory capacity for *S. aureus* (above 80%), however, due to the variability verified among the strains, it is not recommended their use isolated, but they can be used in association with the sensitivity to acriflavine and β-galactosidase tests, increasing the probability of differentiation among the species. The other tests can also be used, but only in association as they presented low discriminatory power.

From the results obtained in this study, it can be suggested that for biochemical identification and differentiation among *S. aureus*, *S. intermedius* and *S. hyicus* species, the sensitivity to acriflavine and β-galactosidase production tests can be used, after a previous positive results in the Gram differential staining, catalase enzyme production, free coagulase and thermonuclease tests. The other tests evaluated in this study could be used as complementation, in order to increase the discriminatory power. Based on the results obtained in biochemical tests, a decision tree was constructed (FIGURE 1), where a minimum number of tests for differentiation of *S. aureus*, *S. hyicus* and *S. intermedius* is suggested.

Conclusions

Among the eight biochemical tests evaluated, the sensitivity to acriflavine and β-galactosidase activity did not present variable results, constituting altogether as the best biochemical tests for differentiation of *S. aureus*, *S. hyicus* and *S. intermedius* species. The other tests can be used as auxiliary tests for identification and differentiation among the species studied, increasing the probability of correct identification.

FIGURE 1 – DECISION TREE WITH MINIMUM NUMBER OF BIOCHEMICAL TESTS FOR IDENTIFICATION AND DIFFERENTIATION AMONG *S. aureus*, *S. intermedius* AND *S. hyicus.*



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