

BIOCHEMICAL CHARACTERISTICS OF TYPICAL AND ATYPICAL *STAPHYLOCOCCUS AUREUS* IN MASTITIC MILK AND ENVIRONMENTAL SAMPLES OF BRAZILIAN DAIRY FARMS

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ABSTRACT

This study reports results on the biochemical characteristics of 274 *Staphylococcus aureus* strains isolated from mastitic milk, hands of milkmen and milking machines. Features included colony morphology on Baird Parker agar (BPA), catalase, coagulase and thermonuclease tests. API-Staph (bioMérieux, France) was used as reference identification system. 39.4% of the colonies on BPA identified as *S.aureus* were atypical. They were more frequent in milk samples (94.4%). All *S.aureus* strains were coagulase positive but intensity of the test varied according to the source: environmental strains were 3+ or 4+, while 17.1% of strains isolated from milk presented only 1+ or 2+ results. The majority of atypical *S.aureus* strains presented a 4+ coagulase result. 2.2% of *S.aureus* strains were thermonuclease negative. Sixteen thermonuclease positive and coagulase positive strains were identified as *S.hyicus*.

Key words: *Staphylococcus aureus*, atypical *S.aureus*, coagulase, thermonuclease, API-Staph, Baird-Parker agar, milk, mastitis, environmental samples

INTRODUCTION

Staphylococcus aureus is a causative agent of mastitis in dairy cattle. When causing mastitis, *S. aureus* can also be found in milk, where high levels of contamination can be rapidly achieved if conditions are favorable. During growth in milk, enterotoxigenic *S. aureus* strains are able to produce thermostable enterotoxins, that, when ingested, cause nausea, vomiting and diarrhea (7).

The method of choice for enumeration of *S.aureus* in most dairy products is the Baird Parker plate count method. This method provides for isolation and enumeration of injured microorganisms without the use of a nonselective enrichment step (12). Baird Parker agar contains sodium pyruvate to protect damaged cells and aid in their recovery, and egg yolk emulsion as a diagnostic agent. Glycine and lithium chloride are the selective agents which suppress the growth of most bacteria, without inhibiting *S.aureus*. Potassium tellurite is reduced to

form grey-black shiny colonies. Typical colonies of *S.aureus* on this medium are 1-1.5mm diameter, with an opaque halo surrounded by a 2-5 mm zone of clearing. The halo is a result of lipase activity and the clearing zone is due to proteolytic action (1,3,6,7,9,10,12).

Atypical colonies of *S.aureus* on Baird Parker agar may occur, and their frequency varies according to the type of food. Baird and Lee (2) reported that selectivity of Baird Parker agar is limited, since only lipolytic and proteolytic *S.aureus* can be easily recognized. According to Benett and Lancette (3) and ICMSF (9), nonlipolytic strains may be frequent in dairy products or milk samples coming from mastitic animals.

Additional diagnostic features are required to confirm *S.aureus* colonies, which include microscopical examination (Gram staining) and biochemical tests, including catalase test, anaerobic utilization of glucose, anaerobic utilization of mannitol, lysostaphin sensitivity and production of thermostable nuclease (TNase)(3). Ready-to-use identification kits, such as the API-Staph system (bioMérieux,

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France) may also be used (10). In most laboratories the confirmation procedures most frequently used to establish the identity of *S.aureus* are the coagulase or/and TNase tests.

Characteristics of *S.aureus* colonies on Baird Parker agar and results of coagulase and TNase tests are critical factors for the proper isolation, identification and enumeration of this microorganism in milk and other dairy products. This study aimed to determine the correlation between morphology of colonies on Baird Parker agar and results for these biochemical features, in *S.aureus* strains isolated from mastitic milk and environmental samples of Brazilian dairy farms.

MATERIALS AND METHODS

Sampling. Milk samples were obtained from mastitic cows of 25 dairy farms located in the south region of Rio Grande do Sul state, Brazil. Mastitis was diagnosed using the California Mastitis Test (CMT), run and interpreted according to Schalm and Norlander, 1957 (13). Environmental samples were collected from milking machines using surface sampling with swabs. Samples from milkmen were obtained by washing one hand of the individual with 0.85% saline in a sterile plastic bag. Samples were transported to the laboratory in insulated boxes and tested within 24h.

Isolation and characterization of *Staphylococcus aureus*. Samples were serially diluted in sterile 0.85% saline and aliquots of 0.1ml were surface plated on Baird Parker agar (Oxoid) and incubated at 37°C for 24-48h. From each plate, five grey to black colonies, with or without halos, were selected and transferred to slants of Tryptic Soy Agar and incubated overnight at 37°C. Each culture was submitted to Gram stain and tested for production of catalase, coagulase and thermonuclease (TNase), according to Lancette and Tatini (10). Regardless results for coagulase and thermonuclease tests, all catalase positive Gram positive cocci were submitted to the API-Staph system (bioMérieux, France) for complete biochemical identification.

RESULTS AND DISCUSSION

Table 1 shows the results of the morphological characterization of 274 colonies isolated from the surface of Baird Parker agar and identified as *Staphylococcus aureus* by the API-Staph system. A significant number of these colonies (108 or 39.4%) did not present the expected typical morphology on Baird Parker agar. Among these, 102 (94.4%) have been isolated from milk samples. Table 1 also shows that the frequency of atypical colonies was much higher among *S.aureus* isolated from milk (40.6%) than that among strains isolated from milking machines or hand samples (26.1%). The frequency of atypical colonies detected in this study was lower than that reported by Wilson *et al.* (14), who observed that none of the *S.aureus* strains isolated from mastitic milk presented halos on Baird Parker agar.

Table 1. Morphological features of colonies of *S.aureus* strains on Baird Parker agar.

origin of strains	morphology		total
	typical ¹	atypical ²	
milk	149	102	251
others ³	17	6	23
total	166	108	274

^{1, 2:} see text for details

³ samples from hands and milking machines

All *S.aureus* strains were positive for the coagulase test. 84.7% of them presented a strong reaction (3+ to 4+), while 15.3% were considered 1+ or 2+ (Table 2). The intensity of reaction varied according to the source of the strains. While all those isolated from milking machines and hands were 3+ or 4+, some of those coming from milk (17.1%) presented only 1+ or 2+ results. Langlois *et al.* (11) also tested *S.aureus* strains isolated from bovine milk for coagulase and observed that all of them were coagulase positive.

Table 2. Results for coagulase and thermonuclease tests of *S.aureus* strains

origin of strains	coagulase			thermonuclease	
	4+ or 3+	2+ or 1+	negative	positive	negative
milk	209	42	0	245	6
others ¹	23	0	0	23	0
total	232	42	0	268	6

¹ samples from hands and milking machines

Forty out of 42 strains with 1+ or 2+ coagulase result presented typical morphology on Baird Parker agar. Besides all of them being isolated from milk, the great majority of these strains were isolated in one single farm (data not shown).

In counterpart, the majority (106/108) of atypical *S.aureus* strains presented a 4+ coagulase result. These results stress Harvey and Gilmour's recommendation (8) about testing for coagulase all colonies on Baird Parker larger or smaller than 1 mm diameter, with or without halo or clearing zones. These authors observed that colonies larger than 1 mm and lacking the halo were those that best correlated with coagulase and thermonuclease results.

There is a controversy concerning the correct interpretation of results of the coagulase test. According to Benett and Lancette (3), 1+, 2+ and 3+ results rarely correlate with results of other criteria for *S.aureus*. However, AOAC (1) and APHA (10) protocols are more flexible: AOAC considers every result (1+ to 4+) as a positive result, while APHA considers only 3+ and 4+ as positive results.

Chang and Huang (5) tested 338 staphylococci strains and observed that when AOAC (1) protocol was used to interpret results, sensitivity and specificity of coagulase test for identification of *S.aureus* were 97.7% (204/213) and 95.2% (119/125), respectively. When APHA (10) recommendations were followed, the sensitivity was very slightly lower (95.8%). However, when BAM (3) was used as reference, sensitivity lowered to 96.7% but specificity increased to 96.8%. In the present study, if only 4+ results were considered to classify a colony as *S.aureus*, 16.8% of the colonies would have been misinterpreted. If the criteria were to consider 3+ and 4+ results, then the rate of false-negative colonies would have dropped to 15.3%. It must be pointed out that seven coagulase positive strains were not confirmed by API-Staph as *S.aureus*. Among them, five presented 3+ or 4+ results for coagulase and two presented a 1+ result.

Among colonies identified by API-Staph as *S.aureus* (274), 268 (97.8%) were TNase positive (Table 2). Similar results were obtained by Bennett *et al.* (4). Working with strains isolated from foods and food ingredients, these authors reported that 93% were TNase positive. However, Chang and Huang (5) reported that 99.5% of the strains produced this enzyme.

In this study, all TNase negative *S.aureus* strains were isolated from mastitic milk, and were coagulase positive (4+). Their morphology on Baird Parker agar was typical. Furthermore, all these strains were isolated in the same dairy farm. Harvey and Gilmour (8) failed to detect TNase negative and coagulase positive *S.aureus* strains in raw milk. Similarly, Langlois *et al* (11) reported that all strains isolated from milk were coagulase and thermonuclease positive.

Although being easily interpreted, TNase test as the unique criteria for identification of *S.aureus* may lead to a high percentage of false-positive results, since other staphylococci may produce this enzyme (7). In this study, 16 strains isolated from mastitic milk, further identified as *S.hyicus* by API-Staph, were TNase positive (data not shown). This result agrees with those reported by Chang and Huang (5), who observed that among 125 non-*aureus* staphylococci, 17 (13.6%) were TNase positive.

Results reported in this study indicate an occurrence of atypical *S.aureus* as high as 39.4% in mastitic milk and environmental samples in Brazilian dairy farms. In milk, atypical colonies were frequently identified as *S.aureus*, but in environmental and hand samples very few of them were identified as such. In spite of the subjectivity of the coagulase test, specially to interpret weak (1+ or 2+) results, the consideration of only 3+ and 4+ results as positive may lead to a high number of false-negative results. The inclusion of additional tests, like the TNase test, is reinforced since many other staphylococci may be coagulase positive. Biochemical testing through ready-to-use systems, such as the one used in this study (API-Staph), may be extremely helpful. In addition,

testing atypical colonies in Baird-Parker agar is mandatory for the correct identification of *S.aureus* in mastitic milk.

RESUMO

Características bioquímicas de cepas típicas e atípicas de *Staphylococcus aureus* isoladas de leite mastítico e de amostras ambientais de fazendas leiteiras brasileiras

Esse trabalho relata as características bioquímicas de 274 cepas de *Staphylococcus aureus* isoladas de leite mastítico, de mãos de ordenhadores e de ordenhadeiras mecânicas. Foram avaliadas as características morfológicas em agar Baird Parker (BPA) e os resultados para os testes de catalase, coagulase e termonuclease. API-Staph foi empregado como sistema de referência para identificação. 39,4% das colônias em BPA identificadas como *S.aureus* apresentaram morfologia atípica, sendo mais frequentes em leite (94,4%). Todas as cepas identificadas como *S.aureus* foram coagulase positivas mas a intensidade da reação variou de acordo com a fonte: cepas isoladas de amostras ambientais foram 3+ ou 4+, enquanto 17,1% daquelas isoladas de leite foram apenas 1+ ou 2+. A maioria das cepas de *S.aureus* com morfologia atípica em agar Baird-Parker apresentaram coagulase 4+. 2,2% das cepas de *S.aureus* foram termonuclease negativas. 16 cepas termonuclease positivas e coagulase positivas foram identificadas como *S.hyicus*.

Palavras-chave: *Staphylococcus aureus*, *S.aureus* atípico, coagulase, termonuclease, API-Staph, agar Baird-Parker, leite, mastite, amostras ambientais

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