Onderstepoort J. vet. Res., 52, 25–29 (1985)

SOME FEATURES OF COAGULASE POSITIVE STAPHYLOCOCCI FROM BOVINE MILK. 1. CARBOHYDRATE METABOLISM: COMPARISON OF CONVENTIONAL TECHNIQUES AND THE API 50 CH SYSTEM

J. A. ERASMUS, Veterinary Laboratory, P.O. Box 625, Kroonstad 9500

ABSTRACT

ERASMUS, J. A., 1985. Some features of coagulase positive staphylococci from bovine milk. 1. Carbohydrate metabolism: comparison of conventional techniques and the API 50 CH system. *Onderstepoort Journal of Veterinary Research*, 52, 25–29 (1985).

When conventional techniques were applied to 84 isolates of *Staphylococcus aureus* from milk samples, it was found that they were all catalase and phosphatase positive and oxidase negative. They all fermented glucose within 24 h and mannitol within 24–48 h when inoculated into Hugh & Leifson's medium, enriched with 1 % horse serum. When they were subjected to the carbohydrates of the API 50 CH system, all metabolized glucose aerobically, but only 85–89 % of the isolates could utilize mannitol aerobically. Because of the difference in the utilization of mannitol observed, the value of the API 50 CH classification in the taxonomy of *S. aureus* becomes questionable. This system could be used as a handy tool, however, when selecting carbohydrates to be used in taxonomical studies.

INTRODUCTION

The 3 species of the genus *Staphylococcus* generally recognized are *S. aureus, S. epidermidis* and *S. saprohyticus* (Baird-Parker, 1963, 1965, 1975; Baird-Parker, Hill, Kloos, Roucus, Oeding & Schleifer, 1976). Of these *S. aureus* is regarded as the main cause of bovine mastitis (Schalm, Carroll & Jain, 1971).

Cowan (1979) described the genus *Staphylococcus* as gram-positive spheres occurring in pairs or in clusters, the cells showing variation in size and in gram-staining properties. Furthermore, the organism is non-motile, non-spore-forming, aerobic and faculatively anaerobic. It produces catalase, but no oxidase, and it attacks carbohydrates by fermentation. In order to be classified as a species of the genus *Staphylococcus, S. aureus* is further expected to produce coagulase and to ferment glucose and mannitol, while acetoin must be an end product of glucose metabolism.

Many schemes have been devised for identifying *S. aureus*. As an example, LeChevallier, Seidler & Evans (1980) employed catalase, oxidase and glucose fermentation as tests in the classification of gram-positive cocci obtained from untreated surface water. An isolate, conforming to those properties which could also ferment mannitol, was designated *S. aureus*. In the veterinary field, Brown, Sandvik, Sherer & Rose (1967) defined *S. aureus* as gram-positive cocci which produce catalase and coagulase and utilize both glucose and mannitol anaerobically. On the other hand, Hess & Stuker (1975) employed glucose fermentation and the coagulation of plasma as the only features to distinguish *S. aureus* from other gram-positive cocci found in bovine udder parenchyma.

In the medical field, McFaddin (1980) indicated that a strong positive coagulase test could be taken as sufficient evidence in the identification of *S. aureus*. *S. aureus* was previously thought to be the only coagulase-producing species of the genus *Staphylococcus*. This assumption can no longer be accepted, as *S. intermedius* and many strains of *S. hyicus* also produce this enzyme (Devriese, 1977; Devriese, Hajek, Oeding, Meyer & Schleifer, 1978; Devriese & Hajek, 1980). The coagulase test on known isolates of *S. aureus* could also result in about 2,5 % of false positive and 1,7 % false negative reactions (Erasmus, 1983). Therefore, plasma coagulation could be taken as an important test in the identification of *S. aureus*, provided that it is used in collaboration with other tests, such as glucose and mannitol fermentation.

For the dissimilation of mannitol by catalase-positive staphylococci and micrococci, Mossel (1962) used Mossel & Martin's (1961) modification of Hugh & Leifson's (1953) test. They (Mossel & Martin, 1961) concluded that mannitol was not attacked if no acid production could be detected after 4 days' incubation at 37 °C. Baird-Parker (1963) formulated a synthetic medium in which acid production from carbohydrates by the staphylococci and the micrococci could be tested. When this medium is employed, acid production could be expected within 5–10 days, a period which is even more impractical than the 4-day incubation in Mossel & Martin's (1961) modification of Hugh & Leifson's (1953) test. Hugh & Leifson's OF (HLOF) medium (Hugh & Leifson, 1953), which was originally developed for the testing of glucose fermentation by the Enterobacteriaceae, differs from Baird-Parker's formulation (Baird-Parker, 1963) in that it contains fewer mineral salts. In addition to yeast extract, 0,2 % tryptose is also added to the former. HLOF medium can further be enriched by the addition of 2 % serum or of 0,1 % yeast extract (Cowan, 1979). Compared with the rate of the above-mentioned media, a faster rate of carbohydrate metabolism could be expected when such organisms are inoculated into HLOF or its modified versions, in which case such a medium would be more suitable for use in the taxonomy of the staphylococci.

A recent development in the field of medical microbiology is the API classification systems of which the API 20 Staph and API 50 CH are examples. Swartz (1984) utilized API 20 Staph to identify proven isolates of *S. aureus* obtained from bovine milk. As only 5 of the 18 isolates tested could be identified as *S. aureus*, Swartz (1984) concluded that the API 20 Staph classification system should not be used in the identification of mastitis causing staphylococci. The API 50 CH classification system contains 49 different carbohydrates, presumably a very practical and handy system for a study of carbohydrate utilization by bacteria.

For the purpose of this investigation, 84 isolates of coagulase-positive cocci were obtained from bovine milk samples. Using conventional techniques, it was first shown that the isolates were all members of the species *S. aureus*. Some of these isolates were subjected to fermentation tests, using HLOF medium as well as a modification thereof in order to find the shortest practical time in which positive results with glucose and mannitol could be read. According to the trial results, all isolates were subjected to fermentation tests with glucose and mannitol. The aerobic metabolism of carbohydrates was tested for in the API 50 CH classification system.

MATERIALS AND METHODS

All milk samples were plated on mannitol salt agar (MSA)* and incubated for 22–24 h at 37 °C. Single colonies selected from these plates were transferred to tryptose blood agar (TBA)* containing 5 % (v/v) bovine

Received 29 October 1984—Editor

TABLE 1 Time taken by *Staphylococcus aureus* to complete oxidation and fermentation reactions in glucose and mannitol in Hugh & Leifson's OF medium

Isolate No.	Carbohydrates without serum														Carbohydrates with serum				
	Oxidation						Fermentation								Oxidation		Fermentation		
	Glucose			Mannitol			Glucose				Mannitol				Glucose	Mannitol	Glucose	Mannitol	
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h	24 h	24 h	24 h	24 h	48 h
1	+			−	−	+	−	−	−	+	−	−	+		+	+	+	+	
2	+			+			−	+			−	+			+	+	+	+	
3	−	+		+			−	+			−	+			+	+	+	+	
4	+			−	+		+				−	+			+	+	+	+	
5	+			+			+				+				+	+	+	+	
6	+			+			+				+				+	+	+	+	
7	−	−	+	−	−	+	−	−	−	+	−	−	−	+	+	+	+	+	
8	−	+		+			−	+			−	+			+	+	+	+	
9	+			+			+				+				+	+	+	+	
10	+			+			+				+				+	+	+	+	
11	−	+		+			−				−	−	−	+	+	+	+	−	+
12	+			+			+				−	+			+	+	+	+	
13	+			+			+				−	+			+	+	+	+	
14	+			+			+				−	+			+	+	+	+	
15	+			+			+				−	+			+	+	+	+	
16	+			+			+				−	+			+	+	+	+	
17	+			+			+				+				+	+	+	+	
18	+			−	+		+				−	+			+	+	+	+	
19	−	+		+			−	+			−	+			+	+	+	+	
20	+			−	+		+				−	+			+	+	+	+	
Positive reactions	15	4	1	15	3	2	13	5	0	2	5	12	1	2	20	20	20	19	1

blood. These plates were also incubated for 22–24 h at 37 °C, after which they were examined for growth and the presence of haemolysis. Cellular morphology and the purity of a culture was determined microscopically after the application of Gram's stain (Preston & Morrell, 1962).

Loopfuls of test organisms were suspended into drops of 3 % H_2O_2 (v/v) on a glass slide to detect the presence of catalase. A test was regarded as positive when gas bubbles evolved immedately.

Oxidase production was tested on filter paper strips, moistened with a fresh 1 % (m/v) aqueous solution of n,n,n',n'-tetramethyl-p-phenylenediamine.** Test organisms taken from TBA were smeared across the surface of the impregnated paper strips by means of a platinum wire needle. A positive reaction was taken as one where a dark-purple colour developed at the junction between the organisms and the paper.

Ability to produce phosphatase was tested on phosphatase agar (Cowan, 1979). Plates inoculated with the relevant organisms were incubated for 22–24 h at 37 °C, after which 0,1 mℓ ammonia solution (sp. gr. 0,880) was placed in the lid of the Petri dish and the culture inverted above it. Phosphatase-positive colonies become bright pink.

Fermentation of glucose was tested for in standard HLOF medium* (Hugh & Leifson, 1953). The medium was modified by the addition of 1 % (m/v) Oxoid No. 1 agar*** only, or by the addition of 1 % (m/v) Oxoid No. 1 agar as well as 1 % (v/v) sterile horse serum. The media were dispersed in 7 mℓ tubes. While still warm a *c.*1,5 cm layer of sterile liquid paraffin was added to the tubes intended for anaerobic reactions. The media were stored at 4 °C. For the test proper, organisms from the same isolate were stab-inoculated into each of 4 tubes, 1 with and 1 without liquid paraffin, 1 set with and the other without serum. An organism which produced acid in the aerobic tube only was designated oxidative (O). Acid production in both the aerobic and the anaerobic tubes was taken as the result of a fermentation (F) reaction.

An OF test, in which glucose was replaced with mannitol in HLOF medium and its modification, was performed following the same general outlines as described above.

Coagulation of rabbit plasma was tested according to the description of McFaddin (1980). A single colony from the 22–24 h growth on TBA was suspended in a 1:10 dilution of fresh rabbit plasma in physiological saline. Coagulation was evaluated after 4 h incubation in a water-bath at 37 °C, and an additional 20 h incubation at room temperature. Only a 3+ or a 4+ reaction (Sperber & Tatini, 1975) was taken as positive.

Single colonies from the 22–24 h growth on TBA were suspended in Voges-Proskauer's (VP) medium for micrococci and staphylococci, as suggested by Baird-Parker (1963). After 48 h incubation at 37 °C, the presence of acetoin was tested for according to Barritt (1936).

The API 50 CH system was inoculated from culture suspensions in phenol-red broth,* containing 1 % horse serum. All reactions were read and recorded after 24 h and again after 48 h incubation at 37 °C. A reaction was taken as positive only when the colour of a suspension changed from red to a definite yellow.

The similarity between 2 organisms was calculated according to the formula given by Stanier, Doudoroff & Adelberg (1972). After similarity coefficients were calculated pair-wise for the different organisms, the data were arranged in a similarity matrix, whence it was transposed into a dendrogram. The computer programme, according to which the calculations were made, was based on the work of Sneath (1972) and Sokal & Sneath (1973).

Results and Discussion

Twenty isolates from the batch of 84 were selected at random and subjected to OF tests in the presence of glucose and mannitol. The results are given in Table 1.

In the absence of serum, oxidation of glucose and mannitol was completed within 72 h. Anaerobic metabolism was completed 24 h later. When serum was added to the media, both the aerobic and the anaerobic reactions were completed within 24 h. Isolate No. 11 was an exception, as a period of 48 h was needed for the completion of mannitol fermentation (Table 1).

OF tests with glucose and mannitol, dissolved in HLOF medium and containing 1 % horse serum, were performed on the 84 isolates. As result of the information in Table 1, these reactions were finally read after 48 hours' incubation at 37 °C. These results as well as those of other phenotypical tests performed on these isolates are summarized in Table 2.

TABLE 2 Features of the 84 gram-positive, coagulase positive cocci

Test	Reaction	
Catalase	+	
Oxidase	−	
Phosphatase	+	
Voges-Proskauer	+	
Glucose (OF)	F	(within 24 h)
Mannitol (OF)	F	(within 48 h)

Clearly, the isolates used in this study were all members of the species *S. aureus* (Table 2), since they fermented glucose and mannitol and produced coagulase, phosphatase and acetoin. When the same isolates were subjected to the aerobic dissimilation of carbohydrates in the API 50 CH system, the isolates could all utilize glucose, thus confirming the results obtained with conventional methods. With regard to mannitol, a positive reaction could be obtained in only 85–89 % of cases (Table 3).

Baird-Parker (1965) employed 14 different carbohydrates in the classification of the staphylococci. These results are also compared with reactions obtained by the 84 isolates under study when tested in the API 50 CH classification system (Table 3). In both these studies *S. aureus* was found to metabolize arabinose, inositol, raffinose and rhamnose. Where Baird-Parker (1965) obtained negative results with cellobiose and salicin, 10–19 % of this particular batch of organisms could utilize these carbohydrates. Fairly comparable results were obtained in the metabolism of lactose, maltose and mannose.

The dendogram which depicts the relationship between the isolates, based on carbohydrate metabolism in the API 50 CH classification system, is given in Fig. 1. At a similarity level of 65 % and above, organisms might be considered as belonging to the same genus, and at a level of 75 % and above they might belong to the same species (Skerman, 1973). The relevant isolates could clearly be classified as a single group with a similarity of

* Biolab Chemicals (Pty) Ltd, P.O. Box 15849, Lynn East, Pretoria 0039

** E. Merck (Pty) Ltd, P.O. Box 3497, Johannesburg 2000

*** Protea Laboratory Services (Pty) Ltd, P.O. Box 5598, Johannesburg 2000

TABLE 3 Carbohidrate utilization of 84 isolates of *S. aureus* in the API 50 CH classification system [figures in brackets are results obtained in conventional tests by Baird-Parker (1965)]

Carbohydrate	% of isolates positive	
D-glucose	100	(100)
D-fructose		
Maltose		(99)
Lactose		(92)
D-turanose		
Galactose	95–99	(54)
D-mannose		(100)
N-acetyl-glucosamine		
Sucrose		
Trehalose		
Amidon		
Ribose	90–94	
Esculin		(3)
Mannitol	85–89	(96)
Glycerol		
Glycogen		
Melezitose	20–84	
Salicin	15–19	(o)
Arbutin	10–14	
Cellobiose		(o)
L-arabinose	5–9	
α-methyl-D-glucocide		
D-taganose		
Sorbitol		
Amygdalene		
Melibiose		
Inulin		
D-raffinose		(o)
β-gentiobiose		
Erytriol		
D-arabinose		(o)
D-xylose		(o)
L-xylose		
Adonitol		
β-methyl-xylocide		
L-sorbose		
Rhamnose		(o)
Dulcitol		
Inositol		(o)
Methyl-D-mannoside		
Xilitol		
D-licose		
D-fucose		
L-fucose		
D-arabitol		
L-arabitol		
Gluconate		
2 keto-gluconate		
5 keto-gluconate		

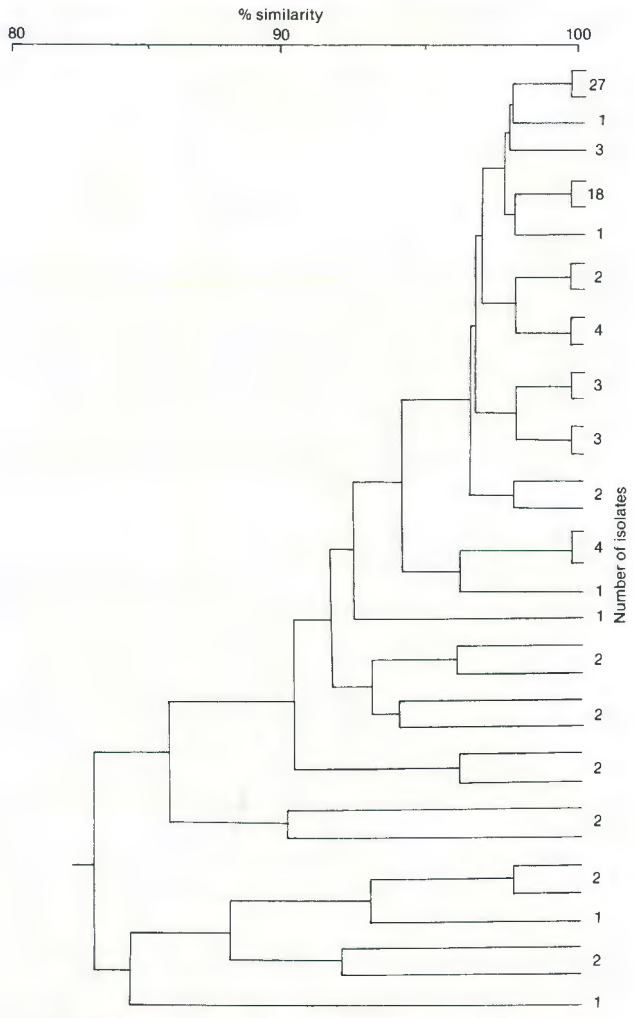

FIG. 1 Dendogram showing the relationship between the different isolates

83 % and more (Fig. 1). According to the above reasoning the 84 isolates could thus all be classified as a single species.

In the case of the metabolism of mannitol, the API 50 CH classification system resulted in negative reactions in which positive reactions with conventional techniques were obtained. If this is the case with an important carbohydrate such as mannitol, one would expect inconsistent result also with other carbohydrates in the API 50 CH classification system, and this would cause doubt as to the value of the latter system in taxonomical work with *S. aureus* (Erasmus, Brand & Martin, 1984). As regards the relationship between the isolates as shown in the dendogram (Fig. 1), the API 50 CH system appears to be a handy tool when selecting carbohydrates to be used in taxonomical work.

From the information given, HLOF medium, enriched with serum, can be used as a quick, reliable method for detecting glucose and mannitol fermentation by *S. aureus*. Final results could be expected within 48 hours' incubation. The API 50 CH system could then be employed when selecting carbohydrates for use in taxonomical work, but not for actual taxonomic investigations on an organism such as *S. aureus*.

ACKNOWLEDGEMENTS

I wish to thank Dr H. van Ark of the division of Biometrical and Datametrical Services for performing the computer analyses and also Mrs M. M. Galbraith for her excellent technical work during this investigation.

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