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THE APPLICATION OF NUMERICAL TAXONOMY IN THE CLASSIFICATION OF STAPHYLOCOCCI FROM BOVINE MILK

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ABSTRACT

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One-hundred-and-two isolates of staphylococci from bovine milk were each subjected to a battery of 19 different tests. With the application of numerical taxonomy these isolates could be classified into 1 genus and 3 different species. Although the majority of the coagulase negative organisms were grouped as 1 species, the biochemical differences within this group indicated that they should belong to at least 2 species. About 50 % of these isolates could be designated *Staphylococcus epidermidis*. Possibly because of the small number of tests, a finer division into different species could not be made.

The coagulase-positive organisms could be divided into 2 species, the smaller group of which consisted of 3 isolates only. If used as the only method for identification, the coagulase test produces false positive results at a rate of about 2,5 % of cases and false negative results at a rate of about 1,7 %.

INTRODUCTION

Using the principles of classical taxonomy, by which some features of a particular organism are conveniently regarded as more important than others, 3 *Staphylococcus spp.*, which include *S. aureus*, *S. epidermidis* and *S. saprophyticus* are recognized (Baird-Parker, 1975; Baird-Parker, Hill, Kloos, Roucus, Oeding & Schleifer, 1976). *S. aureus* is considered to be one of the principal pathogens in bovine mastitis (Schalm, Carroll & Jain, 1971; Anon., 1981). In the past, *S. epidermidis* was regarded as non-pathogenic, but it was recently found to be an important cause of intramammary infections in bovines (Schalm *et al.*, 1971; Hess & Stuker, 1975; McDonald, 1977). *S. saprophyticus* is usually regarded as non-pathogenic (Baird-Parker, 1975).

Sneath (1957 a,b) proposed a mathematical approach to bacterial taxonomy. In this system the relationship between organisms is defined as the number of characteristics which each organism has in common with those with which it is compared, the same degree of importance being attached to all characteristics used in the comparison (Singleton & Sainsbury, 1978). When Hill (1959) applied this technique to *Staphylococcus* spp. from different origins, he could differentiate 5 species, namely, *S. aureus*, *S. afermentans*, *S. lactis*, *S. saprophyticus* and *S. roseus*. Subsequently, new species of staphylococci such as *S. sciuri* (Kloos, Schleifer & Smith, 1976), *S. hyicus* and *S. hyicus* subsp. *chromogenes* (Devriese, Hajek, Oeding, Meyer & Schleifer, 1978) have been described, and *S. epidermidis* now is limited to those organisms formerly classified as *S. epidermidis* biotype I (Brown, 1983).

For the purpose of this paper, 102 isolates which conformed to the basic description of staphylococci obtained from milk samples from various dairy herds, were subjected to a number of tests selected from those described by Baird-Parker (1963). The data thus obtained were classified according to the principles of numerical taxonomy, the aim being to differentiate between different species of staphylococci.

MATERIALS AND METHODS

Isolation and differentiation of Staphylococcus spp.

On their arrival at the laboratory, the milk samples were streaked out on mannitol salt agar* and incubated for 22–24 h at 37 °C. A single colony, selected from the mannitol salt agar plates, was transferred in duplicate to tryptose blood agar* (TBA), containing 5 % bovine blood. Both TBA plates were incubated for 22–24 h at 37 °C, one aerobically and the other anaerobically (Gaspak**). Both plates were subsequently examined for growth and the presence of β-type haemolysis.

Cellular morphology was studied microscopically after the application of Gram's stain (Preston & Morrell, 1962).

Loopfuls of the test organism were suspended in drops of 3 % H_2O_2 on a glass slide to detect the presence of the catalase enzyme. A test was recorded as positive when gas bubbles evolved immediately.

Oxidation and fermentation of glucose was tested for in the standard medium, as proposed by Hugh & Leifson* (1953). The medium was slightly modified by the addition of 1 % Oxoid No. 1 agar.*** The sterile medium was dispensed into 10 mℓ screw-capped bottles and allowed to cool in a slanted position, the slants being such that deep butts as well as slants were obtained. A slant was streaked out and the butt was stabbed with organisms from the same isolate. An organism was designated oxidative when acid production was obtained within 24 h at 37 °C in the slant only. It was designated fermentative if acid production was obtained during the same time and under the same conditions in both the slant and the butt.

Coagulation of rabbit plasma was performed according to the description of McFaddin (1980). A single colony from the 22–24 h growth on TBA (aerobic) was suspended in a 1:10 dilution of rabbit plasma in physiological saline. Coagulation was evaluated after 4 h incubation at 37 °C in a water-bath and an additional 20 h incubation at room temperature.

Single colonies from the 22–24 h TBA growth (aerobic) were suspended in MR-VP broth (Cowan, 1979) and incubated for 72 h at 37 °C. Barritt's method (1936) was used to determine acetoin production.

Acid production by the isolates was performed in 10 mℓ screw-capped bottles containing about 7 mℓ of phenol red broth base* (pH = 7,2–7,4) into which 1 % (w/v) lactose, maltose or mannitol, respectively, was dissolved. The fermentation of these carbohydrates was studied under both aerobic and anaerobic conditions, anaerobic conditions being met by the addition of a layer of sterile liquid paraffin to each of the relevant containers immediately after the addition of a particular isolate. All

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readings were again taken after incubation for 24 h at 37 °C. A positive reaction was recorded only when the colour of the medium changed from red to a definite yellow (pH<6,8).

Numerical taxonomy

The similarity between 2 organisms was calculated, using the formula given by Stanier, Doudoroff & Adelberg (1972) which reads:

$$(a + d)/(a + b + c + d)$$

In this formula

a = number of characteristics positive for both organisms;

b = number of characteristics positive in isolate 1 and negative in isolate 2;

c = number of characteristics positive in isolate 2 and negative in isolate 1;

d = number of characteristics negative in both isolates.

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 dendogram. The computer programme, according to which these calculations were made, was based on the formulae given by Sokal & Sneath (1973) and Sneath (1972).

Results and Discussion

The dendogram indicating the similarity between the different isolates is given in Fig. 1. Clearly, the isolates could all be classified into 2 distinct groups, the smaller

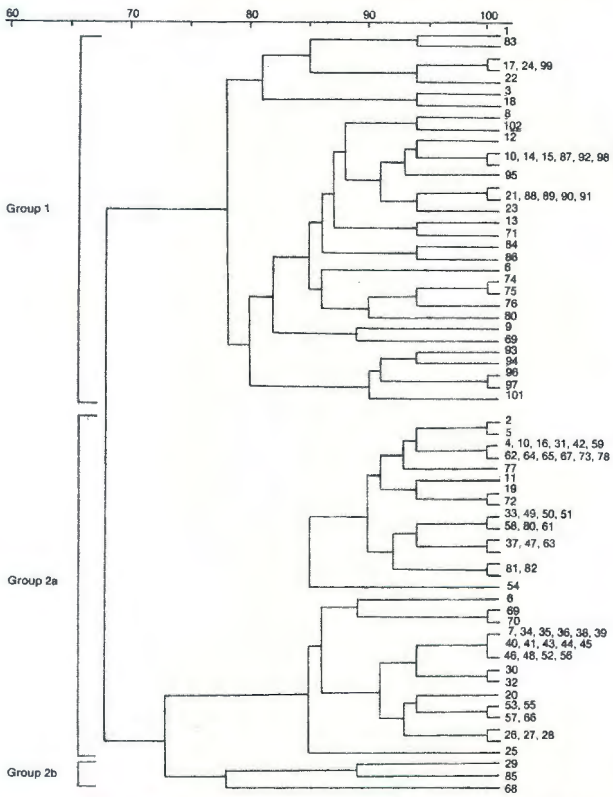

FIG. 1 Dendogram showing the relationship between the different isolates.

group consisting of 40 isolates with a similarity of 79 % and above, and the larger group showing a similarity level of 73 % and above. The similarity for the 2 groups combined was 67 %. 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). According to this reasoning, the 2 groups could be classified into 1 genus and 3 different species.

The biochemical properties of the species thus identified are summarized in Table 1. Evidently all isolates could be cultured in the presence of 7,5 % NaCl. The bacteria were all Gram-positive cocci, which fermented glucose and produced catalase, a definition which conforms to the general description of staphylococci (Baird-Parker, 1975; Cowan, 1979). Group 1 organisms (Fig. 1) consisted of 39 coagulase negative and 1 coagulase positive organism. The coagulase negative staphylococci were previously diagnosed as *S. epidermidis* (Hess & Stuker, 1975). Currently, however, cultures are classified as *S. epidermidis* when they are Gram-positive cocci which produce catalase but not coagulase, ferment glucose but not mannitol anaerobically, and produce slight or no haemolysis (Brown, Sandvik, Scherer & Rose, 1967). The biochemical properties of 20 of the 39 coagulase negative isolates agreed with this definition. Where the mathematical technique resulted in 1 species comprising coagulase negative staphylococci, a study of the actual biochemical properties indicated the presence of more than 1 species in this group.

The most valuable and most accurate tool used in the identification of pathological staphylococci is the coagulase test (Slanetz & Bartley, 1953; Schalm *et al.*, 1971; McFaddin, 1980). In fact, this test could be used as the only procedure for the positive identification of *S. aureus* (McFaddin, 1980). Fifty-eight isolates belonging to Group 2a were found to produce coagulase (Table 1). The bacteria could thus be classified as *S. aureus*. One coagulase negative isolate was also included in this group.

Other important properties of the Group 2a organisms, which distinguished it from those of Group 1 were β-type haemolysis on TBA in both aerobic and anaerobic atmospheres (Table 1). The single coagulase-positive isolate found in Group 1 displayed no lytic properties at all, indicating the possibility of a false positive coagulase test in about 2,5 % of cases. Conversely, a false negative result could be expected in about 1,7 % of cases.

Brown *et al.* (1967) noted that their isolates of *S. aureus* from bovine udders could all utilize mannitol aerobically. Conversely, Hess & Stuker (1975), after examining 201 isolates of micrococcaceae obtained from the parenchyma of bovine udders, concluded that the aerobic breakdown of mannitol was an unsatisfactory criterion for the identification of *S. aureus*. According to the information summarized in Table 1, only 70 % of the isolates from Group 2a, which had been identified as *S. aureus*, could utilize mannitol aerobically, a finding which strongly supports that of Hess & Stuker (1975).

As 3 organisms only are included in Group 2b (Fig. 1), a complete discussion of their properties is not possible.

The application of numerical taxonomy to a fairly large number of samples resulted in a breakdown of 1 genus and 3 species. Possibly because of the small number of tests performed, a finer division within the coagulase negative group could not be made. Such a division, however, could be made after a visual comparison of the biochemical results with the known classification. As was to be expected, the main pathogen isolated was *S. aureus*. With regard to the coagulase-negative group, about half of the isolates only could be designated *S. epidermidis*.

TABLE 1 A comparison of some biochemical reactions of the different *Staphylococcus* spp.

Substrate or test	*Staphylococcus* Group 1[1,2] No. of cultures	%	*Staphylococcus* Group 2a[3] No. of cultures	%	Group 2b No. of cultures
7,5 % NaCl growth	39/39	100	58/58	100	3
Haemolysis (aerobic)	2/39	5	58/58	100	1
Colonies yellow	27/39	69	57/58	98	1
Growth (anaerobic)	39/39	100	58/58	100	3
Haemolysis (anaerobic)	1/39	3	54/58	93	0
Gram-positive	39/39	100	58/58	100	3
Cells round	39/39	100	58/58	100	3
Cells<1µm Ø	5/39	13	27/58	47	0
Cells>1,5µm Ø	6/39	15	0/58	0	2
Catalase positive	39/39	100	58/58	100	3
Glucose (F)	39/39	100	58/58	100	3
Coagulase positive	0/39	0	58/58	100	2
Acetoin produced	1/39	3	51/58	88	3
Lactose (O)	27/39	69	57/58	98	3
Lactose (F)	37/39	95	58/58	100	3
Maltose (O)	25/39	64	56/58	97	3
Maltose (F)	32/39	82	57/58	98	3
Mannitol (O)	6/39	15	40/58	70	2
Mannitol (F)	4/39	10	26/58	45	3

Ø = Diameter
F = Fermentative reaction
O = Oxidative reaction
[1] = Groups referring to Fig. 1
[2] = Coagulase positive isolate excluded
[3] = Coagulase negative isolate excluded

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