Species Differentiation of Group D Streptococci

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ABSTRACT

PAPAVASSILIOU, J. (National University of Athens, Greece), Species differentiation of group D streptococci. Appl. Microbiol. **10**:65–69. 1962.—Three hundred and fourteen strains of group D streptococci were studied by means of a number of tests. The majority of the strains were identified as *Streptococcus faecalis* (83 strains), *Streptococcus faecium* (131 strains), or *Streptococcus bovis* (32 strains). Several strains (47 or nearly 15%) either shared characteristics of two species or were completely atypical. *S. faecalis* and *S. bovis* were more easily identified than *S. faecium*, which is not sharply defined from the other species and could be subdivided into several fermentative types on the basis of fermentation of arabinose, mannitol, sorbitol, glycerol, and sucrose. The value of some characteristics in species identification is discussed. Growth in the presence of potassium tellurite 1:2,500 and in the presence of 6.5% NaCl and fermentation ot arabinose, glycerol, and raffinose are very important tests for the identification of the three species. The reduction of tetrazolium salts, the reduction of litmus milk, and the fermentation of sorbitol may serve as complementary tests for the same purpose. For the differentiation of these three species the "pattern of reactions" is more important than single tests.

During the last ten years, considerable work has been done on the identification and differentiation of faecal streptococci. The work of Shattock (1949, 1955), Skadhauge (1950), Seelemann and Carstens (1951), Sharpe and Shattock (1952), Seelemann (1954), and Barnes (1956) showed that group D streptococci can be divided into four species, namely *Streptococcus faecalis* (or *Streptococcus glycerinaceus*) *Streptococcus faecium*, *Streptococcus bovis*, and *Streptococcus durans*. This nomenclature has been accepted by a number of authors, who studied ecological problems related to group D streptococci and confirmed that a few physiological characteristics are valuable for species identification within group D streptococci (Barnes and Ingram, 1955; Moutoussis, Papavassiliou, and Samaraki-Lyberopoulou, 1957, 1958*a*, *b*; Fewins, Newland, and Briggs, 1957; Buttiaux, 1958; Mieth, 1960). However, Colobert and Morélis (1958) reject the species name *S. faecium*, because many strains of group D streptococci share characteristics of *S. faecalis* and *S. faecium* and others show a completely atypical behaviour in their physiological characteristics.

In previous reports (Moutoussis et al., 1957, 1958*a*, *b*, 1959), we have not fully discussed the value of some physiological characteristics in distinguishing species of group D streptococci. Our results are therefore summarized in the present paper.

MATERIALS AND METHODS

Isolation of strains. A total of 314 strains of serologically grouped streptococci were isolated from 315 faecal specimens of human or animal origin. Methods of isolation have been fully described elsewhere (Moutoussis et al., 1958*b*), but they can be summarized as follows: (i) Inoculation of faecal suspensions in Hajna and Perry sodium azide medium (SF medium[1]) for 48 hr at 45 C; (ii) subculture of positive tubes onto MacConkey agar plates; (iii) selection of several colonies from the MacConkey plates and culture in glucose (1% w/v) broth; and (iv) subcultures were made from each tube of glucose broth onto tetrazolium-agar plates (Barnes, 1956) and onto blood agar containing 1:2,500 potassium tellurite.

One or two isolated colonies were taken from the tetrazolium plates for further study and identification.

Species identification. Methods for identifying species have been detailed elsewhere (Moutoussis et al., 1957, 1958*b*). From a large number of strains examined, 314 were classified serologically as group D streptococci.

The majority of the strains were grouped with sera provided by M. Seelemann, Institute of Milk Hygiene, Kiel, Germany; a potent D-serum of P. M. F. Shattock, University of Reading, England, was also used for a part of our strains. About 50 strains were grouped in Kiel by Prof. Seelemann or in Reading by Dr. Shattock. Finally, another 50 strains were grouped by the author during his stay in the Institute of Milk Hygiene, Kiel.

The following characteristics were studied: haemolysis in human blood agar plates, gelatin liquefaction, growth at 10 C and at 45 C, growth in the presence of 40% bile, growth in the presence of 6.5% sodium chloride in nutrient agar, growth in the presence of 1:2,500 potassium tellurite, reduction of tetrazolium salts at pH 6.0, strong reduction of litmus milk (within

[1] Difco Laboratories, Inc., Detroit, Mich.

24 hr), and fermentation reactions in a medium described by Seelemann (1954) using bromthymol blue as the indicator. With the exception of sorbitol, readings were made and are reported in this study after 48 hr of incubation at 37 C. Late fermentation of sorbitol is also reported, but these late readings do not add much information for the purposes of this study and were excluded from the final report. The use of bromcresol purple as an indicator makes readings necessary after 2 and 7 days (Barnes, Ingram, and Ingram, 1956). The following fermentation reactions were studied: lactose, glucose, arabinose, mannitol, sorbitol, glycerol, sucrose, raffinose, and starch. Only fermentations of arabinose, sorbitol, raffinose, and glycerol have some importance as differential characteristics and they shall be reported in detail. In addition to these four tests, four other tests proved important for the identification of the species within group D streptococci and they will be discussed in the present paper. These tests are: (i) growth in the presence of 1:2,500 potassium tellurite, (ii) growth in the presence of 6.5% sodium chloride, (iii) reduction of tetrazolium studied in tetrazolium agar plates and in tetrazolium broth tubes (Barnes, 1956), and (iv) strong reduction of litmus milk (Shattock, 1955).

Readings of these tests were made within 24 and 48 hr, and after 7 days of incubation at 37 C.

RESULTS

Table 1 summarizes the sources of isolation and the species identification of the 314 strains of group D streptococci used in our study. The results of this table will not be discussed here because they were reported previously (Moutoussis et al., 1958*a*, *b*). The number of strains (17) identified as *S. durans* is too small for further consideration in the present paper. Also, 47 strains which are reported as intermediate or atypical will not be considered in detail here. For 4 strains of *S. faecium*, seven of the eight characteristics were used for species identification. Of the remaining 246 strains, 83 strains were identified as *S. faecalis*, 131 strains as *S. faecium*, and 32 strains as *S. bovis*. These strains will be the subject of this study. Only 3 strains of *S. faecalis* were identified as *S. faecalis* var. *liquefaciens* or *zymogenes*. They behaved typically in the eight tests used for species identification.

The results for the eight characteristics used for species differentiation appear in Table 2. It is clear

TABLE 1. *Group D streptococci isolated from human and animal faeces*

Origin	No. of specimens examined	No. of specimens containing group D streptococci	No. of strains studied	*Streptococcus* species identification				
				S. faecalis	*S. faecium*	*S. durans*	*S. bovis*	Intermediate or atypical strains
Human	117	110	126	73	17	8	14	14
Sheep	55	54	63	4	46	2	5	6
Cow	34	26	26	0	15	0	7	4
Horse	25	24	11*	1	2	0	0	8
Goat	18	16	18	0	10	4	0	4
Pig	10	8	10	1	5	0	1	3
White mice	28	19	20	0	16	1	3	0
White rat	16	16	20	4	12	1	2	1
Rabbit	10	10	10	0	7	0	0	3
Guinea pig	12	10	10	0	5	1	0	4
Total.....	315	293	314	83	135	17	32	47

* Thirteen strains isolated from horses were not grouped serologically and they are not included in this study.

TABLE 2. *Some physiological characteristics of Streptococcus faecalis, Streptococcus faecium, and Streptococcus bovis*

Percentage of strains positive, slightly positive, or negative.

Characteristic	*S. faecalis* (83 strains)	*S. faecium* (131 strains)	*S. bovis* (32 strains)
Potassium tellurite 1:2,500			
Growth	87.9	0	6.2
Poor growth	7.2	3.8	0
No growth	4.7	96.2	93.8
NaCl, 6.5%			
Growth	81.9	97.8	0
Poor growth	4.7	2.2	0
No growth	13.3	0	100.0
Tetrazolium reduction (in agar)			
Reduction	53.0	1.5	3.1
Slight reduction	30.1	0	0
No reduction	16.9	98.5	96.9
Litmus milk			
Reduction in 24 hr	74.7	12.2	6.2
Reduction after 24 hr or no reduction	25.3	87.8	93.8
Fermentation reactions			
Arabinose:*			
Positive	14.4	100.0	90.7
Negative	85.5	0	9.3
Glycerol:*			
Positive	100.0	4.5	3.1
Negative	0	95.5	96.9
Raffinose:*			
Positive	3.6	0.7	100.0
Negative	96.4	99.3	0
Sorbitol:			
Positive*	47.0	12.9	15.6
Late positive (after 48 hr)	22.9	3.8	31.2
Negative	30.1	83.3	53.2

* Acid production within 48 hr. Strains producing acid after 48 hr in the medium used for fermentation reactions are reported as negative for arabinose, glycerol, and raffinose.

that single tests cannot be considered to be conclusive for species identification. Like other groups of microorganisms, e.g., *Enterobacteriaceae*, group D streptococci are a series of interrelated bacterial types, which cannot be sharply divided into species. Many strains are interrelated and they differ in more than one characteristic from the behaviour of the typical species. However, the whole "pattern of reactions" permits the identification of the majority of the strains. The percentage of strains positive or negative in each individual test shows the relative value of each test. These results can be outlined as follows:

1) The potassium tellurite test. This is an excellent test for species identification. A few strains of *S. faecalis* grew poorly or late in the presence of potassium tellurite, 1:2,500. A small percentage of the strains of this species did not grow in the presence of potassium tellurite, 1:2,500, but behaved otherwise as *S. faecalis*. They reduced tetrazolium, reduced litmus milk, fermented glycerol, and did not ferment arabinose. A small number (3.8%) of *S. faecium* strains reduced potassium tellurite slightly, but the other characteristics were typical for *S. faecium*.

2) Reduction of tetrazolium salts. This test is less important than the potasssium tellurite test. A considerable number (16.9%) of *S. faecalis* strains were negative in Barnes' (1956) tetrazolium-agar and an even higher percentage (30.1%) reduced tetrazolium, but colonies were pink (slight reduction). The triphenyltetrazolium chloride (TTC) broth (Barnes, 1956) gave better results for *S. faecalis*, but this medium cannot be recommended because many strains of *S. faecium* also reduced tetrazolium. Readings can be made in 24 or 48 hr because prolongation of the incubation period at 37 C for several days does not influence the degree of reduction of tetrazolium. The test is generally very delicate and depends essentially on the pH of the medium which must be exactly 6.0 (Morélis and Colobert, 1958).

3) Strong reduction of litmus milk. This test might be valuable if evaluated with the other tests. However, a high percentage of *S. faecalis* strains (25.3%) did not reduce litmus milk within 24 hr. Although a small number of *S. faecium* strains did not reduce litmus milk after 7 days of incubation, the majority of the strains were able to reduce it after 48 hr, and even 12.2% reduced litmus milk within 24 hr. The readings of this test should be made at 24 hr only and they must be compared with the other tests, because of the relatively high percentage of strains which do not behave typically.

4) Growth in the presence of 6.5% NaCl. The test is very important for the identification of *S. bovis*. It is very interesting that some recently isolated strains (13.3%) of *S. faecalis* do not grow in the presence of 6.5% NaCl and they may be confused with atypical *S. bovis* strains. However, these strains of *S. faecalis* are able to grow in nutrient agar containing 6.5% NaCl after a second or third transfer on blood agar.

5) Fermentation of arabinose. Although all strains of *S. faecium* fermented arabinose, some strains of *S. bovis* failed to ferment it and 14.4% of the *S. faecalis* strains fermented arabinose.

6) Fermentation of glycerol. Glycerol was fermented by all strains of *S. faecalis*, but only by 4.5% of *S. faecium* strains and 3.1% of the strains of *S. bovis*. It seems to be a very important test in species identification.

7) Fermentation of raffinose. It is very important for the identification of *S. bovis*, as very small numbers of *S. faecalis* (3.6%) or *S. faecium* (0.7%) ferment raffinose.

8) Fermentation of sorbitol. This fermentation reaction is less important than the other tests reported in the present study. Only 47% of the strains of *S. faecalis* fermented sorbitol within 48 hr, whereas 12.9% of the *S. faecium* strains and 15.6% of *S. bovis* strains fermented sorbitol within 48 hr. Strains fermenting sorbitol after 48 hr are very common among *S. faecalis* but also among *S. bovis* and the sensitivity of the test cannot be increased essentially by taking into consideration fermentation of sorbitol after 7 or 14 days. The test should be considered for species identification together with the other tests.

Discussion

The methods of isolation used in this study are more selective for the isolation of *S. faecium* than for other species. Many strains of *S. bovis* or *S. durans* were probably lost during isolation. The reasons for using these methods have been detailed elsewhere (Moutoussis et al., 1958*b*). More recent work (Buttiaux, 1958; Kjellander, 1960) showed that the lower azide concentration used in Hajna and Perry's medium and also primary incubation at 37 C were useful for the isolation of a greater number and types of faecal streptococci. Mieth (1960) demonstrated that the types of group D streptococci isolated from faeces of pigs depend on the type of media used for primary isolation. These observations are very important for studies concerned with the distribution of group D streptococci in faeces. However, these observations cannot play any rôle in the results of the present study which is concerned with the behaviour of our strains and the identification of species within group D streptococci.

The identification of species of group D streptococci is not always easy. However, a number of strains behave typically as *S. faecalis*, *S. faecium*, or *S. bovis*. Of the 314 strains in our study, 47 (nearly 15% either) shared characteristics of two species (intermediate strains) or were completely atypical. *S. faecalis* and *S. bovis* were easy to identify, as they demonstrated

more or less typical behaviour. *S. faecium* is less homogeneous in physiological characteristics. By the use of five fermentation reactions, Moutoussis et al. (1959) described six fermentative types of *S. faecium* (Table 3). Type III is very active in fermentation reactions and usually reduced litmus milk within 24 hr. This type is more or less related to *S. faecalis*, but gives negative potassium tellurite and tetrazolium reduction tests. Type VI ferments only arabinose, glucose, and lactose and is closer to *S. durans*. This type does not reduce litmus milk. Between these two extremes are found more typical representative types of the species. *S. faecium* is not sharply distinguished from the other species, but the rejection of this species as suggested by Colobert and Morélis (1958) is not justified. Deviations from the behaviour considered as "typical" are present in many groups of microorganisms. Many striking examples have been reported by persons working with *Enterobacteriaceae*. The repetition of some tests for strains of group D streptococci, which behave atypically, seems to be valuable, especially after serial transfers on suitable media (blood agar). Some strains of *S. faecalis*, which did not grow initially in the presence of 6.5% NaCl, gave a positive reaction after one or more transfers onto blood agar. I have no valid explanation for this phenomenon and I have not studied the relationship, if any, to the medium of isolation which contains a relatively high concentration of sodium azide.

The value of individual tests in species identification has been already discussed. Our results are more or less similar to those obtained by other authors. The reduction of tetrazolium gave less satisfactory results in our hands than those reported by Barnes (1956). Also, strong reduction of litmus milk was not a typical characteristic (*see* Shattock, 1955). The fermentation of glycerol was in agreement with the results obtained by Seelemann (1954) and Mieth (1960), but differed from the results of Wahl and Meyer (1956), who found that only 65% of strains of *S. faecalis* ferment this sugar. However, we prefer the name *S. faecalis* to *S. glycerinaceus* (Seelemann, 1954), because we believe that no species identification should be done on the basis of a single test. The fermentation of arabinose is a valuable test, although some exceptional strains of *S. faecalis* may ferment arabinose. Fermentation of sorbitol is a less satisfactory test. Our results are in agreement with those of Mieth (1960).

TABLE 3. *Fermentative types of Streptococcus faecium (Moutoussis et al., 1959)*

Type	Arabinose	Mannitol	Sorbitol	Glycerol	Sucrose	Total no. of strains studied
I	+	+	−	−	+	45
II	+	+	+	−	+	11
III	+	+	+	+	+	6
IV	+	−	−	−	+	19
V	+	+	−	−	−	7
VI	+	−	−	−	−	42*

* Type VI includes 36 strains isolated from sheep faeces. This type was rarely found in human and pig faeces. There is no strict relationship between the fermentative type of *S. faecium* and the source of isolation. The distribution of the fermentative types in human and animal faeces are detailed in the paper of Moutoussis et al. (1959).

The general conclusion of this paper is that species identification and differentiation of group D streptococci is possible, but as Shattock (1955) says, "although group D streptococci comprise the several well defined species and variants described here, not all group D streptococci can be assigned a species name. It has been acknowledged that there are no clear-cut lines of demarcation between the various species within this group and strains are frequently encountered which cannot be given a species name, and for the present must remain anonymous."

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