Studies in the Differentiation between Human and Animal Pollution by means of Faecal Streptococci

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With a Note on the Standardization of the Janus Green Milk Test

SUMMARY: The isolation of faecal streptococci from the excreta of man, cattle and sheep was investigated. The methods compared were (a) heat, (b) tetrathionate, (c) tellurite, (d) thallium salts. The tellurite method proved highly efficient, isolating 97 % of strains from human, cow and sheep faeces. A survey of the properties of strains isolated was made. Differential tests based on (a) heat resistance and (b) reducing properties were devised; these divided the streptococci isolated by the tellurite method into groups, some of which were characteristic of the source. The information available seems capable of distinguishing many strains obtained from man from those derived from cows or sheep, and it is suggested it may be of use in tracing sources of pollution in water, milk and other foodstuffs.

No satisfactory method exists at present for distinguishing faecal pollution by human sources from that by other animal sources. The chief organism used in proving faecal pollution, namely *Escherichia coli* type I, may arise from either source (see Rep. Minist. Hlth, Lond. 1940). Faecal streptococci have also been advocated as confirmatory evidence of pollution, but the significance of different types has been little investigated. The nature of the types isolated from faecal material depends to some extent on the method of isolation. The strains isolated by the two methods proposed in the Ministry report, namely the heating method and the tellurite method, are often different. Other methods of isolation are also possible. Cooper, Baker, Elliot & Wood (1942) reported the isolation by tetrathionate media, and Cooper & Linton (1947) showed the resistance of these organisms to thallium acetate. The use of sodium azide was investigated by Allen, Pierce & Smith (1953).

The present paper describes the results of experiments which compared these various methods (except the use of azide), and a more extended investigation of the streptococci obtained from human, cow and sheep faeces by the tellurite method. As a result it appears that a high percentage of strains have properties which enable their source to be determined with considerable certainty. The faecal streptococci, therefore, in contrast to *Escherichia coli* may prove of value in tracing pollutions in water, milk and other foodstuffs.

The isolation of streptococci by tetrathionate broth and by heat (60° for $\frac{1}{2}$ hr.) was compared by Cooper, Baker, Elliot & Wood (1942), and though equally successful from human faeces, showed that heat destroyed many strains in cattle excreta. That investigation, and a further one in 1950, revealed that three-quarters of the streptococcal strains isolated from cattle and sheep produced green-yellow discoloration on heated blood agar. Less than one-

quarter of the human strains did this; for the media used see Cooper, Mayr-Harting & McLachlan (1950). However, variability of the results on repeated subculture and with changes in the media led us in this investigation to study reducing properties instead of the production of peroxides.

SURVEY OF ISOLATION METHODS

Thirty specimens of faeces from human, cow and sheep sources were concentrated in three different media, namely:

(a) *Tetrathionate broth* as used for the isolation of *Salmonella paratyphi* B from faeces (Cooper, Wood, Elliot, Caswell & Small, 1942).

(b) *Potassium tellurite medium*: a modification of the medium of Harold (1936) for isolation of streptococci from water was used; potassium tellurite, final concentration 1/5000 in 0.5 % lactose, 0.5 % peptone water buffered at pH 7.6 with 0.5 % potassium phosphate.

(c) *Thallium acetate medium* which contained 0.5 % peptone, 0.5 % lactose, 0.5 % anhydrous dipotassium phosphate and thallium acetate to make final concentration 1/2000 (Cooper & Linton, 1947).

Method. A loopful of excreta was emulsified in 10 ml. sterile water; filtered through four layers of muslin, the filtrate mixed and with a Pasteur pipette 10 drops inoculated into tubes of each of the three media. Plate cultures were made after 24 hr. on to MacConkey agar (0.5 % sodium taurocholate) and incubated for a further 24 hr. Streptococcal colonies were picked and subcultured on 10 % heated blood agar until pure. A well-defined colony was distributed in Bactopeptone broth and then incubated at 37° for 16 hr. The resulting growth was examined microscopically and subjected to a series of biochemical tests to study the following properties:

Haemolysis. Shattock's modification (1949) of Brown's technique.

Gelatin liquefaction. Stab cultures observed over 14 days at room temperature.

Action in litmus milk at 37°. The term 'strong litmus reduction' was applied to cases where the dye was totally reduced in the depths of the tube in 24 hr. with or without colour change at the surface. The medium (5 ml.) was in screw-cap bottles (1 oz. McCartney), incubated with the stopper tight and not shaken.

Survival at 60° for 30 min. A sample (0.5 ml.) of culture in Bactopeptone broth was transferred into a Widal tube (65 × 9 mm.) and placed in a water-bath at 60° with the level of culture well below the water and heated for 30 min. The tubes were then transferred to another water-bath at 37° and kept for 2 hr. Subcultures were then made on 10 % heated blood agar and plates incubated for 48 hr. and examined for growth.

Growth at pH 9.6. Method of Shattock & Hirsch (1947).

Inhibition by 6.5 % sodium chloride. Method of Abd-el-Malek & Gibson (1948).

Inhibition by 0.1 % methylene blue. A loopful of culture was inoculated in 5 ml. sterile skimmed milk containing 0.1 % methylene blue and incubated in a water-bath at 37°. The results were read after 24 hr.

Growth on 40 % ox bile medium. Abd-el-Malek & Gibson (1948).

Starch hydrolysis. Poured plate cultures made by using 1 ml. culture and melted 0.2 % starch agar cooled to 45°. Hydrolysis tested with iodine solution after 5 days at 37°.

Sodium hippurate hydrolysis. Ayers & Rupp (1922).

Sugar fermentation reactions were observed in peptone water to which was added a sterile solution of 0.5 % sucrose, lactose, raffinose, inulin, mannitol, or 1 % glycerol, and phenol red.

RESULTS

Isolation methods

By using the three selective media 195 strains of streptococci were isolated from the ninety specimens of faeces. The relative number of strains recovered by each medium in accordance with its value as a method of isolation is shown in Table 1. *Tetrathionate broth*, though often successful with human faeces, failed rather significantly in concentrating streptococci from sheep and bovine faeces. *Proteus vulgaris*, micrococci and yeasts were usually prevalent.

Table 1. *Incidence of streptococci isolated by three different methods*

		Method of isolation						Total strains isolated
		Tetrathionate		Tellurite		Thallium acetate		
		No. of strains	% of success	No. of strains	% of success	No. of strains	% of success	
Faeces								
Human	30	18	60	30	100	24	80	72
Bovine	30	1	3	28	93	28	93	57
Sheep	30	7	23	29	96	30	100	66
Total	90	26	29	87	96.7	82	91.1	195

The strains isolated by each method usually differ in properties and have therefore been regarded as distinct, though originating from the same specimen.

Potassium tellurite medium proved to be highly selective in concentrating streptococci from human and animal faeces (97 % of specimens). The advantage of a concentration of 1/5000 is that it helps to diminish *Proteus vulgaris* and fungi which are often predominant in animal faeces. Fleming (1932) discovered that some proteus strains resist 1 : 8000 potassium tellurite and that 1 : 5000 did not affect faecal streptococci. Additional experiments with solid media revealed that faecal streptococci from man, cow and sheep multiplied on nutrient agar or (10 %) heated blood agar containing 0.2 % potassium tellurite.

Thallium acetate medium may be considered equal to tellurite medium in securing heavy pure growth of streptococci from animal faeces but it failed in a considerable number of cases with human faeces.

Strains isolated

By using the three methods of isolation it was possible to obtain at least one streptococcus strain from every specimen of faeces. Usually strains with different characters were recovered from the same specimen when treated with different methods. Strains which showed all the classical characteristics of named species have been so designated. In an endeavour to classify the other streptococci into convenient groups the first eight characters presented in Table 2 were taken as the basis for differentiation. According to this method, 40 % of human-derived streptococci were grouped as *Streptococcus faecalis*, a specific name given by Andrewes & Horder (1906) and firmly established by Dible (1921) and Sherman (1938). Typical *S. faecalis*, as described in Table 2, did not exist among streptococci derived from cow and sheep faeces. *S. durans* was also only recovered from human faeces and represented 4 % of human strains. *S. faecalis* var. *liquefaciens* and *S. faecalis* var. *zymogenes* accounted for about 29 % of the human-derived streptococci. The liquefaciens variety represented a group of 14 % of bovine and 18 % of sheep strains. The zymogenes variety was not encountered among the bovine organisms but formed 6 % of streptococci from sheep. Many investigators have recovered the liquefaciens and zymogenes variants from human faeces, milk, ice-cream, dried milk and cheese, but there seems no evidence from the available literature that they have been noticed before in bovine or sheep faeces.

Human, bovine and sheep faeces share in common several groups of streptococci which differ from the typical *Streptococcus faecalis* in one or more essential criteria and usually other secondary ones. Since the object of the present work is the subdivision of streptococci from various sources into groups convenient for differential purposes, the name 'Atypical faecalis' designated I, II, III, IV and V was applied to those strains which differed from the typical faecalis in one, two or three standard characters. These undoubtedly would include most of the unclassified and the so-called *S. faecalis* 'variants' and 'enterococcus-like' organisms recorded by several investigators.

Only one strain of *Streptococcus bovis* was encountered among the human-derived collection. Unlike the other *S. bovis* strains this one failed to ferment starch. Orla-Jensen (1942) observed that human strains of *S. bovis* did not hydrolyse starch. *S. bovis* represented 28 % of streptococci recovered from cow dung; none was found in sheep dung. The presence of *S. bovis* in small numbers in the human intestine had been recognized by many investigators; Sharpe (1948) encountered thirty-seven *S. bovis* strains out of 340 streptococcal strains recovered from the faeces of human infants. This high incidence may be attributed to the common presence of this organism in cow's milk which may be incompletely sterilized for infant feeding. The recovery of *S. bovis* in only 28 % of bovine-derived strains is a result different from that of Ayers & Mudge (1923) who considered *S. bovis* as the predominating streptococcus in bovine intestine. This difference in findings may be due to the methods of isolation used or to the different criteria for identification. None

Table 2. *The physiological characters of streptococci from human, bovine and sheep faeces*

Origin of streptococci	Classification	No. of strains	No. of strains recovered by each method	Haemolysis	Gelatin liquefaction	Strong litmus milk reduction	Mannitol	Sucrose	Raffinose	Survival at 60° for 30 min.	Growth at pH 9.6	Growth in 6.5 % NaCl	Starch hydrolysis	Growth in 0.1 % M.B. milk	Sodium hippurate hydrolysis	Inulin	V. Glycerol	V. Sorbitol
Human faeces	<i>S. faecalis</i> (typical)	29	a. 5 b. 15 c. 9	-	-	+	A.	V.	-	+	+	+	-	+	+8 -3	-	A.	A.
	<i>S. faecalis</i> var. <i>liquefaciens</i>	11	a. 3 b. 5 c. 3	-	+	+	A.	A.	-7 +4	+	+	+	-	+	+	-	A.	A.
	<i>S. faecalis</i> var. <i>zymogenes</i>	10	a. 2 b. 3 c. 5	+	-7 +3	+	A.	A.	-	+	+	+	-	+8 -2	+9 -1	-	A.	A.
	<i>S. durans</i>	3	b. 1 c. 2	+	-	-	-	-2 +1	-	+	-	+2 -1	-	-	-2 +1	-	-	-
	<i>S. bovis</i>	1	b.	-	-	-	A.	A.	A.	-	-	-	-	-	-	A.	-	-
	Atypical faecalis I.	11	a. 6 b. 4 c. 1	-	-	-	A.	A.	-	-	+	+	-	+w.	+9 -2	-	A.	A.
	Atypical faecalis II.	7	a. 2 b. 1 c. 4	-	-	-	A.	A.	-	+	± V.sl.	+	-	-	+w.	-	-	A.
	<i>S. bovis</i>	16	b. 10 c. 6 c. 3	-	-	-	-	+14 -2	A.	-	-	-	+	-	-	A.	-	-
	<i>S. faecalis</i> var. <i>liquefaciens</i>	8	b. 5 c. 3	-	+	+	A.	A.	-	+	+	+	-	+	+4 -4	-	A.	A.
	Atypical faecalis I.	12	a. 1 b. 6 c. 5	-	-	-	A.	A.	-	-	+	+	-	+7w. -5	-8 +4	-	-7 +5	-7 +5
Bovine faeces	Atypical faecalis III.	10	b. 4 c. 6 c. 6	-	-	-	A.	A.	A.	-	+	+	-	-7 +3	-	-	+6 -4	+8 -2
	Atypical faecalis IV.	8	b. 2 c. 6 c. 6	-	-	-	A.	A.	A.	+	± V.sl.	+5 -3	+	+4w. -4	-5 +3	-	-	A.
	Atypical faecalis V.	3	b. 1 c. 2 c. 2	-	-	-	-	A.	-	+	+	+	-	-	+w.	-	-	-
	<i>S. faecalis</i> var. <i>liquefaciens</i>	12	a. 1 b. 6 c. 5	-	+	+	A.	+10 -2	-	+	+	+	-	+	+7 -2	-	+10 -2	+10 -2
	<i>S. faecalis</i> var. <i>zymogenes</i>	4	b. 2 c. 2 c. 4	+	+2 -2	+	A.	A.	+2 -2	+	+	+	-3 +1x	+w.	+2 -2	+2 -2	A.	A.
	Atypical faecalis II.	26	a. 4 b. 9 c. 13	-	-	-	A.	A.	-	+	+14 +12 V.sl.	+	-	-	-	-	-22 +4w.A.	+19 -7
	Atypical faecalis III.	15	b. 9 c. 6 b. 3	-	-	-	A.	A.	A.	-	+	+	-	-	-	-	-	-
	Atypical faecalis V.	9	a. 2 b. 3 c. 4	-	-	-	-	A.	-	+	+	+	-	+	-5 +4w.	-	-	-

All these strains grew on 40 % bile broth and fermented lactose. Abbreviations: a., tetrathionate method; b., tellurite method; c., thallium acetate method. A., acid production; V.sl., very slight growth; w., weak (growth, reduction or fermentation, etc.); x., reaction exception to rule.

of our *S. bovis* strains was capable of surviving a temperature of 60° for 30 min. This is contrary to Sherman's findings (1937) and agrees with observations made by Shattock & Mattick (1943). The discrepancy may be due to differing amounts of peroxide formed in varying media, as a lethal factor additional to the temperature.

SURVEY OF DIFFERENTIAL TESTS FOR INDICATING SOURCE

The tests used to distinguish species within this group have increased in number and variety in recent years, and this has resulted in a multiplicity of recognizable types. Which of these types are worthy of being called species, or indeed, which of the tests employed should be regarded as diagnostic and which as only showing minor variants is still disputed. We are not here primarily concerned with classification but with the value of some of these tests as an indication of the sources of the strains concerned.

Choice of method of isolation

The method of isolation itself determines some of the characteristics of the strains isolated. The use of 1/5000 potassium tellurite followed by subculture to MacConkey agar (0.5 % sodium taurocholate) isolated a high proportion (97 %) of the bile-resistant enterococci from faeces of man, cow and sheep. This work requires extension to other sources of pollution such as birds, pigs, dogs, cats, rabbits, rats, etc.; nevertheless, the strains thus isolated probably represent the predominant organisms likely to gain access to water or milk supplies. Isolations by this method were therefore made from 350 specimens of faeces (130 human, 110 cow, 110 sheep). Bactopeptone broth cultures (16 hr., 37°) of the purified strains isolated were prepared for examination by different tests (343 strains).

Choice of differential tests

Earlier work had suggested the value of heat-resistance tests and of tests for oxidizing or reducing ability. The difference between human and animal strains was of a quantitative nature and best demonstrated on freshly isolated strains. With a view to avoiding, if possible, prolonged quantitative measurements it was decided to utilize a dye which indicated different degrees of reduction, and Janus Green in milk was therefore compared with the previously used litmus milk. The heat test was made a little more severe (63° for 30 min.), and the recovery of strains was tested not only in a rich medium (10 % heated blood agar), but also on McLeod's tellurite medium (Anderson, Happold, McLeod & Thompson, 1931).

Heat-resistance tests. A sample (0.5 ml.) of culture was transferred into a Widal tube (65 × 9 mm.); the tube placed in a water-bath at 63° with the level of the culture well below the water and heated for 30 min. The tube was then transferred to another water-bath at 37° and kept for 2 hr. With a 4 mm. diameter loop one loopful was subcultured: (i) to a (10 %) heated blood agar

and the plate incubated for 48 hr. after which it was examined for growth; (ii) to McLeod's tellurite medium and the plate incubated at 37° for 48 hr. and examined for growth.

Janus Green milk test. Janus green B (dimethylsafranin azodimethylaniline; a Gurr preparation; batch no. 1041, see p. 189) was dissolved in sterile glass-distilled water to form a 1 % solution. The dye solution was heated in a water-bath at 80–100° for 15 min. and left to cool. It was then added to sterile separated milk to bring the final concentration to 1/10,000 and the medium distributed in 5 ml. amounts in screw-capped bottles. One loopful (4 mm. diameter) of inoculum from the broth culture was transferred to a screw-capped bottle of medium and incubated along with a non-inoculated bottle of medium as control in a water-bath at 37°. Readings were made after 16 hr. to record the rate of reduction of the dye in the milk culture.

Litmus milk reduction test. One loopful (4 mm. diameter) of broth culture was put in 1 % litmus milk and the bottle incubated in a water-bath at 37° for 16 hr. after which the changes and rate of reduction of litmus in the milk culture were recorded. These technical procedures were conducted on adequate numbers of human, cow and sheep-derived strains in parallel, so that the conditions under which these strains were tested may be considered comparable.

RESULTS

The tellurite method isolated streptococci from 127 (97·6 %) of 130 samples of human faeces; from 106 of 110 samples (96·3 %) of cow faeces and from 110 of 110 (100 %) samples of sheep faeces, which when examined by the above procedure revealed the findings stated in Table 3. It was found that when Janus Green B underwent reduction in a milk culture, a series of changes in colour occurred in the following order: blue → mauve → violet → dirty red → wine red (quinonoid) → rosy pink → pale pink → colourless. The Janus Green milk test was considered positive when the colour of the dye reached after 16 hr. at 37° was the quinonoid red (semi-reduced form) or any shade following it towards the leucoform of the dye. The rosy pink colour, however, was that most often observed in positive Janus Green milk tests. On the other hand, a negative result was taken as indicated by any culture in which the colour lay between the blue and the dirty red shades. To avoid confusion between 'dirty red' and 'quinonoid red', the cultures were shaken before examination, thus allowing atmospheric oxygen to restore the violet shade to cultures which had not been reduced to the quinonoid red form.

The Janus Green milk test revealed 85% positive results among strains of human origin, and only 19·4% among those of animal origin. The results obtained with the litmus milk test showed that 72·4% of human-derived strains and 46·2% of the animal-derived ones were capable of reducing the dye to the colourless form during the same period of incubation.

Janus Green has shown itself to be a better indicator than litmus of the relative degree of reduction of streptococcal milk cultures. Unlike litmus, Janus Green does not change colour so readily with pH changes of the medium

which makes it a more suitable indicator for the present purpose of indicating degrees of reduction. In comparing the results of the two tests on the same strains under the same conditions, not only did the Janus Green test show superiority in detecting a higher percentage of strong-reducing strains among the human-derived cultures, but it also gave less than half the percentage of reducing strains among the animal-derived cultures than did the litmus test. About 50 % of cultures which did not reduce Janus Green within 16 hr. did so on incubation for a further 24 hr. This suggests that differences among excremental streptococci in their rate of Janus Green reduction are mainly due to differences in ability to reduce the dye rather than differences in their susceptibility to the antiseptic effect of the dye.

Table 3. *Incidence of positive and negative biochemical tests shown by streptococci of different origin*

Origin	Total no. of strains	Heat-resistance test (63°, 30 min.)	Heat and tellurite resistance test	Janus Green milk test	Litmus milk test
Human	127	+ 115 (90.5 %) – 12 (9.5 %)	+ 97 (76.4 %) – 30 (23.6 %)	+ 108 (85 %) – 19 (15 %)	+ 92 (72.4 %) – 35 (27.6 %)
Cow	106	+ 22 (20.7 %) – 84 (79.3 %)	+ 0 (0 %) – 106 (100 %)	+ 21 (19.8 %) – 85 (80.2 %)	+ 44 (41.5 %) – 62 (58.5 %)
Sheep	110	+ 35 (31.8 %) – 75 (68.2 %)	+ 0 (0 %) – 110 (100 %)	+ 21 (19 %) – 89 (81 %)	+ 56 (50.9 %) – 54 (49.1 %)

The heat-resistance test revealed that 90.5 % of the human and 26.3 % of the animal strains were thermotolerant. Heating at 63° was the temperature of choice. It gave a better differential test than that at 60°. It may be worth recording that some human strains were found to resist heating at 63° for 1 hr. and some for 90 min.

Faecal streptococci, when subjected to the heat-test fell into three categories: (a) strains killed by heat; (b) strains which survived the heat test but were subsequently not able to survive on the inhibitory medium; (c) strains which survived the two processes. Most of the heat-resistant strains from human faeces (84.3 %) qualified as category (c), i.e. survived both processes; none of the animal strains which survived the heat test belonged to this category.

The inhibition of growth of heat-treated cells on a 0.04 % potassium tellurite medium seems to be due to a weakness in the resistance of these cells acquired as a result of heat treatment, rather than to a bactericidal action of potassium tellurite. These same strains when unheated produced profuse black growth on McLeod's medium. That 0.04 % potassium tellurite is an important factor in the disinfection process was shown by the observation that a medium containing 0.02 % of that chemical generally supported the growth of heat-treated cells.

Significance of differential tests

When the strains examined are classified according to the two heat-resistance tests and the Janus Green reduction test into the six possible groups (Table 4), then the source of strains falling into some of these groups seems to be indicated

clearly. Groups I and II were derived entirely from human sources; other animal sources accounted for 98.5 % of group III and 91 % of group IV. Strains with the characters of groups V and VI were less characteristic of a particular source.

Table 4. *Biochemical grouping of faecal streptococci*

Group	Janus Green B milk test	Heat- resistance test	Heat and tellurite resistance test	Incidence						Total 343
				Human- derived strains (127)		Cow-derived strains (106)		Sheep-derived strains (110)		
				% of		% of		% of		
				No.	group	No.	group	No.	group	
I	+	+	+	84	100	Nil	0	Nil	0	84
II	—	+	+	13	100	Nil	0	Nil	0	13
III	—	—	—	2	1.5	68	50.4	65	48.1	135
IV	—	+	—	4	8.8	17	37.6	24	53.3	45
V	+	+	—	14	46.7	5	16.7	11	36.7	30
VI	+	—	—	10	27.8	16	44.4	10	27.8	36

Conclusions

Whether strains with these characteristics will be as diagnostic of their source when a wider field is surveyed, remains to be seen. Our results suggest that with carefully devised tests it should be possible to distinguish human pollution from animal pollution by means of the faecal streptococci. The application of such tests after the tellurite method of isolation should be further investigated, and also compared with results by other methods of isolation such as the glucose + yeast-extract + sodium azide method advocated by Allen *et al.* (1953). The final identification of a strain by the use of most of the tests used in Table 2 will sometimes give more conclusive results. The characterization of a strain as typical *Streptococcus faecalis* seems to indicate a human origin. A starch-positive *S. bovis* points, on the other hand, very definitely to an animal origin.

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Note on the Standardization of the Janus Green Milk Test

Because the dye Janus Green B (dimethylsafranin azodimethylaniline: a Gurr preparation, batch no. 1041) was manufactured mainly for staining purposes and no actual account was recorded of its impurities or ash content, it was found necessary to standardize fresh batches of the dye against this original preparation. The dye preparations examined were: (1) Janus Green B (Gurr preparation, batch no. 351); (2) Janus Green (British Drug Houses Ltd. preparation, batch no. 188914); (3) Original batch, namely Janus Green B (Gurr preparation batch no. 1041). The depth of the blue colour produced by these dye preparations in sterile milk showed that the B.D.H. batch (no. 188914) and the original Gurr batch (no. 1041) both produced a deep blue colour in final concentrations of 1/10,000; but the other Gurr batch (no. 351) only showed a faint bluish tint in a final concentration reaching 1/5000.

Preparation of Janus Green milk. A 1% solution was prepared from each dye batch in sterile glass-distilled water, heated in a water-bath at 80–100° for 15 min. and then cooled. Bulk skimmed milk sterilized by steaming, was divided into three separate portions which were treated as follows:

The first portion. From batch 1041 (Gurr), the dye was added to the milk to produce a 1/10,000 concentration, and then distributed in 5 ml. amounts in

screw-capped bottles. This batch formed the original Janus Green B milk to be considered as the basis of standardization.

The second portion. The milk was divided into four parts. From the 1% solution of the batch no. 183914 (B.D.H.) the dye was added, in different proportions, to bring the final concentrations in these milks to 1/12,000, 1/10,000, 1/8000, 1/5000, respectively. The milk and dye was then distributed into screw-capped bottles in 5 ml. amounts and labelled.

The third portion was treated as the second portion except that dye batch no. 351 (Gurr) was used instead of the B.D.H. batch.

Test cultures. Sixty cultures of excremental streptococci from the collection isolated by the tellurite method (20 human, 20 cow and 20 sheep) were sub-cultured in Bactopeptone broth and incubated at 37° for 16 hr.

Method. One loop (4 mm. diam.) inoculation was made from each of the 16 hr. Bactopeptone broth cultures into bottles of Janus Green milk from the three different batches. The milks were then incubated in a water-bath at 37° with uninoculated controls. Readings were made after 16 hr.

Results. The original Janus Green B milk test (containing 1/10,000 Janus Green B batch no. 1041, Gurr) revealed that twenty-eight cultures (16 human, 12 animal) reduced the dye to the quinonoid red or pale pink form within 16 hr. at 37°, i.e. gave a positive test. In the remaining thirty-two cultures the dye remained oxidized (blue) or was only partially reduced (mauve, violet, dirty red), i.e. gave a negative test.

With the other dye+milk preparations, only the 1/8000 with batch no. 183914 (B.D.H.) milk test reproduced the same results as of the original test.

The 1/10,000 (B.D.H.) milk test, on the other hand, gave nine 'false-positive' results, while the 1/5000 concentration of the same batch showed an over-poising action in eleven cultures (i.e. false-negative results). Janus Green B (batch no. 351, Gurr) in the various concentrations used in milks, was reduced by all the strains involved in this investigation, either to the pale pink or the leucocolour.

From these results it may be assumed that in order to maintain its differential value between excretal streptococci, the Janus Green milk test should either be prepared in 1/10,000 final concentration from the 'Gurr preparation batch no. 1041' or in 1/8000 final concentration from the 'B.D.H. preparation batch no. 183914'. Every batch should be standardized, and not all are suitable. A small stock of B.D.H. preparation no. 183914 has been reserved to standardize new batches of Janus Green.

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