A SIMPLE COLORIMETRIC METHOD FOR THE ESTIMATION OF RELATIVE NUMBERS OF LACTOBACILLI IN THE SALIVA[1]

MARSHALL L. SNYDER

Hygienic Laboratory, University of Michigan, Ann Arbor, Michigan

This paper includes the results of an attempt to develop a simple colorimetric method for the estimation of the relative numbers of lactobacilli in the saliva as a supplementary procedure for the quantitative techniques (1, 2) used in the study of dental caries. The test was based on the assumption that since caries activity is closely correlated with the numerical rise and fall of lactobacilli in the saliva, it should be possible to demonstrate a corresponding increase and decrease in the amount of acid produced in a selective carbohydrate medium inoculated with definite amounts of saliva. Thus, significant color changes in selected time intervals could be interpreted roughly in terms of lactobacilli per c.c. of saliva as established by an adequate number of control plate counts. The present data embodies a large series of tests on specimens of saliva sent to the School of Dentistry of the University of Michigan for estimation of numbers of lactobacilli.

METHOD AND MATERIAL

The essential requirement for such a method was a medium selective for the acidogenic organisms, especially the lactobacilli, in the saliva and so colored that shifts in the indicator could easily be detected. Beef infusion agar to which glucose or lactose and an indicator were added was found to be very satisfactory since acid tomato-peptone agar (2) masked the color of the indicators used. Since the medium to be sufficiently selective must have an initial acidity of at least pH 5.0, the number of indicators was at once limited. After many trials with brom-cresol-green (pH 3.8–5.4), brom-phenol-blue (pH 3.0–4.6), benzene-azo-a-naphthylamine (pH 3.7–5.0), and a-naphthylamine-azo-sulfanilic acid (pH 3.5–5.7), it was found that brom-cresol-green was the most satisfactory because the color changes were easy to follow and the indicator was stable at sterilizing temperatures.

Using specimens of saliva from 38 selected caries-free children and 134 freshman medical students, it was found that "shake" tubes, to which measured amounts of saliva could be

[1] Supported in part by a grant from the Horace E. Rackham School for Graduate Study.

added at 45°C followed by immediate cooling of the medium and daily observation for 4 days at 37°C, gave more consistent results than either streaked plates or stabbed slants. These preliminary tests established a definite correlation between intensity and time of color change and estimated numbers of lactobacilli in the respective specimens.

Changes in the color of the indicator were recorded as 0 for no difference compared with uninoculated controls and 4 for a complete shift from the blue-green to the yellow. Intermediate stages were estimated as 1, 2 or 3 (*fig.* 1). However, to avoid the confusion in estimating degrees of intensity, significant or positive changes were considered to be only those tubes in which green was no longer the dominant color and determined as either 3 or 4. This point on the colorimetric scale for brom-cresol-green is approximately pH 4.2–4.4, and was selected because pure cultures of streptococci, yeasts, and staphylococci did not alter the color below this limit whereas cultures of lactobacilli, especially the S form, caused a complete shift in 48 hours.

To supplement the preliminary observations a survey by clinical and quantitative methods of the incidence of caries in a large number of children in a "mottled enamel" district in Texas[2] was welcomed. The specimens of saliva were collected in sterile bottles at different public schools and shipped by air mail to Ann Arbor where definite quantities were sampled before "shake" tube cultures were made. In this manner lactobacillus counts[3] were determined independently and were not compared with the results of the color tests until after the latter were discarded. All the results reported in this paper were determined with "shake" tubes which contained approximately 5–7 c.c. of the acid glucose or lactose agar containing brom-cresol-green as the indicator and inoculated with either 0.1 c.c. or 0.2 c.c. of saliva.

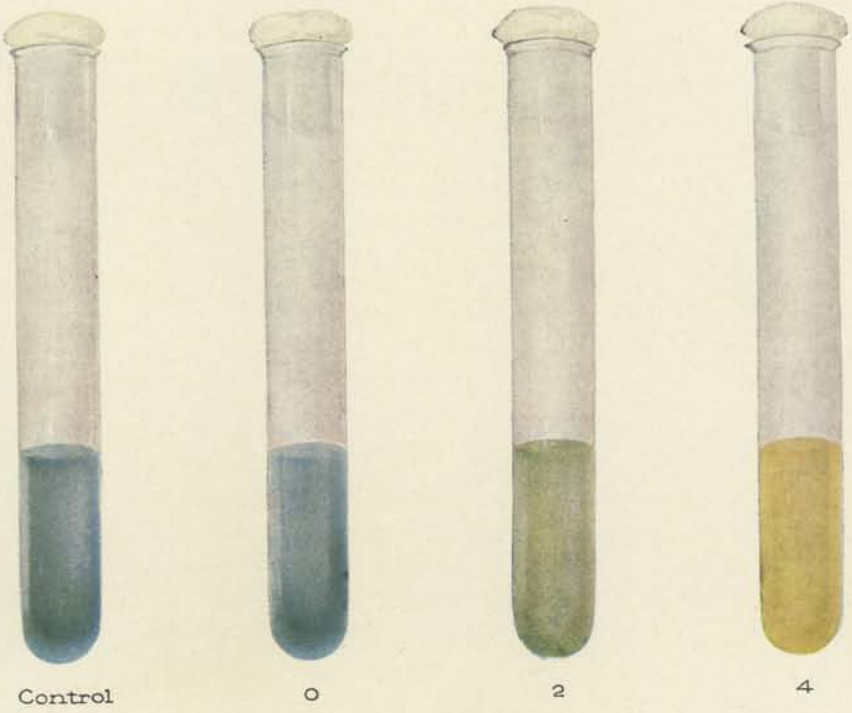
RESULTS

The significant color changes every 24 hours for 4 days with percentages of positive results in relation to independent lactobacillus counts are recorded in Tables I, II and III. Table I contains the data when 0.1 c.c. was inoculated into 2 per cent glucose infusion agar (pH 5.0) with bromcresol-green as the indicator. The lactobacillus counts were arbitrarily grouped.

Table I shows that in 24 hours only 1 specimen of saliva induced significant color change but in 48 hours the number of cultures showing distinct acid production increased proportionately with the estimated numbers of lactobacilli. At this time, however, only 2 of 158 specimens containing less than 1,000 lactobacilli per c.c. of saliva were recorded as positive. The same trend was observed at 72 and 96 hours with less clear cut features except the almost uniformly complete color shifts with lactobacillus counts above 20,000 per c.c. of

[2] Conducted for the U. S. Public Health Service by Dr. H. T. Dean and associates. Dr. Philip Jay was a consultant in this study.

[3] The author is indebted to Dr. Philip Jay of the School of Dentistry for the quantitative lactobacillus counts.



Changes in brom-cresol-green glucose agar (pH 5.0) in 48 hours inoculated with 0.2 c.c. saliva.

saliva. The failure to interpret color changes at 48 hours with lactobacillus counts between low and high numerical levels emphasized that acid production was not always dependent on numbers of lactobacilli and was correspondingly influenced by many factors, some of which will be mentioned later in this paper.

Similar tests were carried out with an increase of the inoculum to 0.2 c.c. of saliva. The results are given in Table II.

TABLE I

Comparison of acid tomato-peptone agar plate counts of lactobacilli in the saliva with color changes in brom-cresol-green glucose agar (pH 5.0) "shake" tubes inoculated with 0.1 c.c. saliva

LACTOBACILLI/C.C. SALIVA (TOMATO AGAR PLATE COUNTS)	NO. SPECIMENS	TIME IN HOURS							
		24		48		72		96	
		No. Positive*	Per Cent	No. Positive	Per Cent	No. Positive	Per Cent	No. Positive	Per Cent
0	114	0	0	1	0.88	3	2.6	8	7.0
0– 100	14	0	0	0	0	3	21.4	4	28.6
100– 1,000	30	0	0	1	3.33	19	63.4	26	86.7
1,000– 5,000	44	0	0	18	40.8	39	88.7	43	97.8
5,000– 10,000	25	0	0	14	56.0	23	92.0	24	96.0
10,000– 20,000	22	0	0	10	45.5	17	77.3	19	86.5
20,000– 30,000	19	0	0	14	73.7	18	94.7	19	100
30,000– 50,000	18	0	0	11	61.2	18	100	18	100
50,000–100,000	32	0	0	30	93.7	30	93.7	32	100
100,000–	45	1	2.2	40	89.0	42	93.5	43	95.7
	363								

* Positive changes included only those tubes in which green was no longer the dominant color, and recorded as 3 or 4.

In this series the same failure to produce significant amounts of acid in 24 hours was noted, but within 48 hours the number of cultures showing distinct color change was greatly increased. However, less than 2 per cent of the specimens having under 100 lactobacilli per c.c. of saliva were recorded as positive. Nearly uniform complete changes were observed with specimens containing over 100 lactobacilli per c.c. of saliva in 72 and 96 hours. It was apparent that increasing the inoculum from 0.1 c.c. to 0.2 c.c. of saliva caused a larger number of positive reactions in 48 hours with respect to lactobacillus popula-

TABLE II

Comparison of acid tomato-peptone agar plate counts of lactobacilli in the saliva with color changes in brom-cresol-green glucose agar (pH 5.0) "shake" tubes inoculated with 0.2 c.c. saliva

LACTOBACILLI/C.C. SALIVA (TOMATO AGAR PLATE COUNTS)	NO. SPECIMENS	TIME IN HOURS							
		24		48		72		96	
		No. Positive*	Per Cent	No. Positive	Per Cent	No. Positive	Per Cent	No. Positive	Per Cent
0	102	0	0	2	2.0	4	4.0	11	11.0
0– 100	18	0	0	0	0	6	33.3	12	67.0
100– 1,000	28	0	0	7	25.0	24	85.8	27	96.5
1,000– 5,000	50	0	0	21	42.0	42	84.0	47	94.0
5,000– 10,000	15	0	0	9	60.0	14	93.5	15	100
10,000– 20,000	18	0	0	12	67.0	17.5	94.5	18	100
20,000– 50,000	25	0	0	20	80.0	25	100	25	100
50,000–100,000	24	1	4.2	22	91.7	24	100	24	100
	280								

* Positive changes included only those tubes in which green was no longer the dominant color, and recorded as 3 or 4.

TABLE III

Comparison of acid tomato-peptone agar plate counts of lactobacilli in the saliva with color changes in brom-cresol-green lactose agar (pH 5.0) "shake" tubes inoculated with 0.2 c.c. saliva

LACTOBACILLI/C.C. SALIVA (TOMATO AGAR PLATE COUNTS)	NO. SPECIMENS	TIME IN HOURS							
		24		48		72		96	
		No. Positive*	Per Cent	No. Positive	Per Cent	No. Positive	Per Cent	No. Positive	Per Cent
0	29	0	0	1	3.5	2	6.9	2	6.9
0– 100	11	0	0	1	9.1	2	18.2	3	27.3
100– 1,000	22	0	0	2	9.1	14	63.7	19	86.5
1,000– 5,000	15	0	0	1	6.7	12	80.0	14	93.5
5,000– 10,000	7	0	0	3	43.0	7	100	7	100
10,000– 50,000	16	0	0	13	81.5	16	100	16	100
50,000–100,000	16	0	0	11	68.8	16	100	16	100
100,000–	26	0	0	21	80.8	26	100	26	100
	162								

* Positive changes included only those tubes in which green was no longer the dominant color, and recorded as 3 or 4.

tion. A few tests with 0.3 c.c. and 0.5 c.c. of saliva did not materially change the percentages already listed. As in the previous series there was the same objection of attributing acid production in 48 hours solely to numbers of lactobacilli, although the difference approached a minimum with a longer incubation period.

It was thought that another carbohydrate might give better differentiation; consequently, lactose was substituted for glucose in the acid infusion agar. This medium was inoculated with 0.2 c.c. of the respective specimens of saliva; the results are given in Table III.

Although this series was much smaller than the others, the relation of color changes to respective lactobacillus counts seemed to have much wider numerical limits; that is, of specimens with counts under 5,000 lactobacilli per c.c. of saliva only 6.5 per cent were positive, whereas all specimens of saliva with more than 5,000 lactobacilli per c.c. were strongly positive in 72 hours.

DISCUSSION

Since it has been established that caries remains absent or inactive with few or no lactobacilli in the saliva and becomes active when these forms appear in constant or increasing numbers, it is possible to predict caries activity by quantitative estimation of the numbers of lactobacilli in the saliva (3). At present, methods for estimating their numbers are limited to the laboratory; consequently it was attempted to develop for the practicing dentist a simple colorimetric test which would give essentially the same information easily and rapidly.

It was recognized that acid glucose broth cultures of saliva, although furnishing a valuable aid in detecting the presence of lactobacilli, gave no clue to the original numbers inoculated. In the same manner, final pH values of these broth cultures, as shown by Boyd, Zentmire and Drain (4), would in no way reflect the numbers in the inoculum. However, the use of a selective acid agar medium containing an indicator in "shake" tubes indicated in preliminary tests a correlation between color changes in this medium after inoculation with measured amounts of saliva and the estimated numbers of lactobacilli in the respective specimens. This relationship was partially confirmed in a large series of color tests controlled by separate lactobacillus counts. Thus, it was possible to select by negative color change

in 48 hours those specimens containing less than 1000 lactobacilli per c.c. of saliva with about 4 per cent error, but with increasing numbers of lactobacilli it was difficult to interpret color changes with numbers of lactobacilli until high numerical levels were reached where only a small percentage of specimens of saliva failed to induce color change in 48 hours. The failure to secure consistently positive results even with large numbers of lactobacilli in the saliva made clear the difference between what was being sought and actually what was happening; namely, the color changes reflected the total acid produced by acidogenic organisms in the saliva rather than numbers of lactobacilli. Any attempt, therefore, to correlate or interpret these findings in terms of caries or numbers of lactobacilli must include a consideration of many factors some of which may be briefly discussed.

In contrast to the quantitative method which gives relative but constant results for numbers of lactobacilli, despite all the errors that influence surface plate counts, the test tube procedure is one in which all the organisms of the saliva are incorporated into a selective but different and partially anaerobic medium. Although pure cultures of yeasts and streptococci did not cause significant changes, their influence on acid production in symbiosis with the lactobacilli must be considered. Fosdick and Hansen (5) have already shown that an enormous increase in lactic acid occurred when yeasts were added to cultures of lactobacilli. Furthermore, there are many variants among the oral lactobacilli (6), and our experience showed that the S forms produced acid more readily and in greater concentration than the R types. This correlation between fermentative ability and colony forms was recently established by Tracy (7) with another species of lactobacillus, *L. plantarum*. Hence, it would seem possible that symbiotic relationships and types of lactobacilli might influence the amount of acid produced by the organisms of the saliva. This possibility suggests the interesting speculation whether the amount of acid produced by the oral flora might not be a more accurate index of caries activity than numbers of lactobacilli as illustrated by the many exceptions to the production of significant amounts of acid in 48 hours or even longer periods by specimens of saliva with varying numbers of lactobacilli. This matter is of special interest since there is little information on the rate of cavity formation in terms of num-

bers or varieties of acidogenic bacteria in the mouth. An extensive program to test this point is being conducted and will be reported at a later time.

SUMMARY

Changes in color in selected time intervals of brom-cresol-green glucose or lactose infusion agar (pH 5.0) in "shake" tubes after inoculation of 0.1–0.2 c.c. of saliva were correlated with quantitatively estimated numbers of lactobacilli in the respective specimens. It was found possible to select by negative color change in 48 hours with 4 per cent error those specimens having less than 1000 lactobacilli per c.c. of saliva, but it became increasingly difficult to interpret color shifts directly in terms of lactobacilli in the saliva. The possibility of direct application in the study of dental caries is discussed.

REFERENCES TO LITERATURE

(1) RODRIGUEZ, F. E., *J. A. D. A.*, 17: 1711, 1930.
(2) HADLEY, F. P., *J. D. Res.*, 13: 415, 1933.
(3) JAY, P., *Bacteriology and Immunology of Dental Caries* in *Dental Science and Dental Art* edited by Gordon, S., (Philadelphia, Lea and Febiger, 1938).
(4) BOYD, J. D., ZENTMIRE, Z. AND DRAIN, C. L., *J. D. Res.*, 13: 433, 1933.
(5) FOSDICK, L. S. AND HANSEN, H. D., *J. A. D. A.*, 23: 401, 1936.
(6) HADLEY, F. P., BUNTING, R. W. AND DELVES, E., *J. A. D. A.*, 17: 2041, 1930.
(7) TRACY, R. L., *J. Bact.*, 33: 467, 1939.