From the Dental Research Station of the Royal Medical Board, Vipeholm Hospital, Lund, Sweden. (Head: B. E. GUSTAFSSON, M.D.)

Sampling Methods for the Determination of the Number of Lactobacilli in the Oral Cavity

By

R. GRUBB and B. KRASSE

The relationship between caries activity and the number of lactobacilli in the saliva has for many years been a point of central interest in bacteriologic investigations of the etiology of caries. This is because the lactobacilli belong to the most active acid-forming bacteria in the oral flora, and further because most research workers, though not all, have found a good correlation between the number of lactobacilli in the saliva and caries activity. (Readers interested in the survey of the literature on this problem are referred to APPLETON 1944.) For these reasons it was thought worth while studying the occurrence of lactobacilli in caries-active and inactive dietary groups in the Studies of Dental Caries at Vipeholm Hospital, Lund.

The method most widely used in investigations of this type is to allow the patient to chew a piece of paraffin wax and to use the saliva thus stimulated for bacterial culture (HADLEY 1933). This method which requires co-operation of the subject, cannot, however, be used in investigations on young children, for example. Neither could it always be used for the present material (mental defectives), because a fair percentage (in some groups more than 60 per cent) of the patients were not able to co-operate satisfactorily for the collection of such samples. It was therefore obvious that passive sampling methods had to

be used. A search was therefore made for a practical passive sampling method.

Another problem that presented itself was that a considerable number of the patients had so many *Candida* in the mouth that it was difficult or impossible to count the number of lactobacillus colonies on the "selective" tomato-agar substrate. By the addition of sodium azide to the substrate (DIAMOND, 1950), the growth of *Candida* was inhibited without any demonstrable effect on the growth of lactobacilli (see KRASSE & MÖLLER, 1951).

Material and Methods

Medium and Incubation

Difco's "tomato juice agar" was used throughout as a starting material. The agar content was increased to 2.5 per cent. It was found useful first to melt the agar by itself with a portion of the distilled water (DEWAR & PARFITT, 1951). The pH was adjusted to 5.0 with lactic acid. During the first part of the investigation (the »swab» method) use was made of a medium without any addition of sodium azide, during an intermediate period (the "tooth brush" method) use was made of some plates with 0.01 per cent sodium azide and some without any such addition. During the latter part (the "spoon" method), only medium containing sodium azide was employed.

The reference strains of lactobacilli, obtained from the American Type Culture Collection (*L. acidophilus, L. casei, L. plantarum, L. arabinosus, L. brevis*) and from the National Collection Type Cultures, England (*L. odontolyticus I* and *II*, *L. acidophilus*, *L. casei*) were found to grow on the medium used.

The plates were incubated aerobically for 4 days at 37° C.

Identification

The lactobacilli were identified by the appearance of the colonies and by the absence of catalase, as demonstrated by pouring 10 per cent H_2O_2 over the colonies and watching for any development of gas. Further, every type of colony on the

plates was examined for its microscopic morphology. In doubtful cases, colonies were subcultured in broth and on blood agar.

Other macroscopically visible colonies rarely appeared on this medium. They usually consisted of strains of streptococci, staphylococci, *G. tetragena* or *Sarcina*. No colonies of gram-negative rods were observed.

Sampling times

All samples were collected between 10 a.m. and 11.30 a.m. (the patients had their breakfast at 7.30 a.m., lunch at 11.30 a.m.).

Sampling and Treatment of the Samples

I. The "swab" method.

Deposits on the surface of the teeth and saliva from the vestibule were collected by means of a sterile swab. The swab was then placed in a test-tube containing 2.5 ml. sterile saline. The tube was subsequently shaken by hand. A second swab was then dipped in the tube and streaked on the tomato agar plate.

II. The "tooth brush" method.

The patients' teeth were brushed, after which the tooth brush was rinsed in 100 ml. normal saline. This was repeated 3–4 times. The suspension was then shaken with glass beads for 15 minutes, after which 0.2 ml. were spread on the tomato agar plate. After having been thoroughly washed, the tooth brushes were disinfected by placing them in an iodine-alcohol-water solution with an iodine concentration of about 1/10,000 [1] for two days and subsequent storage in 0.5 per cent sodium thiosulphate solution for a day (for removal of the iodine) and careful rinsing in distilled water.

III. The "spoon" method.

Deposits on the dental surfaces were collected by means of a Ward's carver. The carver was wiped against the edge of a

[1] According to BÜRGER & KALIES (1944), this concentration kills spores of *B. subtilis*.

cup-shaped impression in specially manufactured rectangular stainless steel plates (3.5×1 cm.). The impression, which was 5.5 mm. in diameter, was filled to the brim with plaque material. The amount of debris in this impression weighed about 40 mg. (mean value of 10 different spoonfuls=42 mg. Variation 38–46 mg.) As a rule, it was necessary to scrape the buccal surfaces of several teeth of the upper jaw before sufficient material was obtained. Sometimes it was necessary to scrape all the (remaining) teeth. Dental calculus was avoided.

Once filled, the steel plates were immediately deposited in centrifuge tubes containing 1 ml. of 1 per cent glucose broth and 10–15 glass beads.

As soon as possible after the sample had been collected, the test tube was shaken in a shaking machine for 10 minutes. Afterwards, tomato agar plates were immediately inoculated with 0.1 ml. from a 1 ml. pipette containing at least 0.2 ml., after which the inoculum was spread by means of a bent glass rod, all in accordance with T. SNYDER's (1947) recommendations.

The carver and the steel plates were sterilized at 180° C.

IV. Hadley's method.

The plates were inoculated with 0.1 ml. of a 1 : 10 dilution of paraffin-stimulated saliva.

Classification and Evaluation of the Results of Culture

The results obtained by the "swab" method were classed as positive or negative, according to the presence or absence of lactobacilli. In the other methods, the number of lactobacillus colonies up to 400 per plate was counted.

In view of the extensiveness of the investigations, it was practically impossible to examine more than one dilution of a sample from one and the same patient several times. In some of the plates in which the number of colonies exceeded 400, growth was so dense that differentiation beyond this limit was no longer possible.

In the classification of the results, use was made of a logarithmic scale with a class interval of 1. The material was thus divided into the following three classes:

Class I = 0– 10 colonies per plate
II = 11–100 colonies per plate
III = more than 100 colonies per plate.

Classed by the number of lactobacilli per millilitre of saliva in investigations using the Hadley method, Class I corresponds to 1–1,000, Class II to 1,100–10,000 and Class III to more than 10,000 lactobacilli.

Results

Sampling by the "swab" method presented no practical difficulties, but the "tooth brush" method had certain technical disadvantages, such as the disinfection of tooth brushes and the mechanical shaking of a large number of 150 ml. test tubes. By the "spoon" method about 30 samples could be collected in an hour. Sterilisation of the instruments and the shaking of the tubes were simple procedures.

Comparison of the Results of Samples Obtained by Active and Passive Sampling Methods

The swab method was only used for preliminary qualitative studies. This method was therefore not compared with the quantitative Hadley method. As to the tooth brush method, which was also abandoned, the results soon showed that about 60 per cent of the patients were lactobacillus negative, a figure suggesting lack of agreement between this method and the Hadley method. The results of parallel studies of samples collected by Hadley's method using paraffin-stimulated saliva and of samples collected by the spoon method were compared. A series of patients who were able to co-operate actively in the collection of the samples were studied on 3 different occasions

at 10–14 days interval. On two of the occasions the samples were removed with the aid of a Ward's carver before the patients chewed paraffin wax and on one occasion the paraffin-stimulated samples were collected before those collected by the carver. It was found that the order of collection of samples had no influence on the results. The results recorded on all 3 occasions are therefore accounted for together. The results of the 127 parallel investigations are given in Table 1. The difference is expressed as class intervals with a logarithmic value of 1.

Table 1. *Comparison between the results obtained by the Hadley method and by the spoon method (size of difference between two values)*

		No. of cases
Difference	$\leqq 1$ class interval	120
	$> 1 \leqq 2$ class intervals	7
	> 2 class intervals	0
		127

In 120 of the 127 pairs of investigations, the results of the two methods differed from one another by less than 1 class interval.

The results were analysed further (Table 2) by comparing the number of cases in which one method or the other gave the greater number of lactobacillus colonies.

Table 2. *Number of cases in which Hadley's method (=H) gave a higher value than the spoon method (=S) and vice versa*

H = S	H > S	H < S	Total
46	40	41	127

Most of those 46 pairs of values that were identical belong to groups "0" and "more than 400" lactobacillus colonies.

No systematic difference was observed between the Hadley method and the cup method, the higher value being found just as often by one method as by the other.

In those 7 cases in which the difference between the values obtained by the Hadley method and the spoon method was more than 1 class interval, the value obtained by the Hadley method was higher in 3 of the cases. Here, again, no systematic difference was found between the two methods.

Variability

Purely methodologic inaccuracy of the methods such as slight variation in the amount of inoculum and slight variation in the conditions of growth offered by different preparations of media can cause a certain variation in the results. This variability can be assessed by using plates prepared on different days and counting the number of colonies growing on inoculation of the plates with a volume of 0.1 ml. of a known dilution of a lactobacillus culture. The broth dilution, which was kept at −5° C. was calculated to contain about 2,500 bacteria per ml. (It might be mentioned that storage of the strains used at −20° C resulted in rapid, severe decrease in viability.) Before the inoculation the test tube was shaken for 10 minutes in a shaking machine. The numbers of colonies which grew up were as follows: 208, 132, 228, 254, 238, 280, 257, 278, 228, 264. The mean value was 237 and 9 of the 10 determinations fell within the range of ±20 per cent of the mean value. The bacteria appeared to have retained their viability unchanged for at least 1 month.

As observed by HADLEY in 1933, the number of lactobacilli in the mouth of one and the same person can vary widely from one occasion to another, even though the interval be short. This variability was demonstrated in the present material because all of the patients were examined by the spoon method on three different occasions at intervals of 10–14 days. Throughout the time of the investigation every patient was under the same experimental conditions. All the samples were taken between 10 a.m. and 11.30 a.m. Table 3 shows the figures recorded for 208 persons. The class interval which was equal to 1 on the logarithmic scale was used as a measure.

Table 3. *Comparison between the results of samples removed on different occasions from one and the same person (3 examinations)*

Largest difference	≦ 1 class interval	= 137	65.9
» »	> 1 ≦ 2 class intervals	= 63	30.3
» »	> 2 class intervals	= 8	3.8
		208	100 %

This variability was found to be roughly equal in the five dietary groups examined and they were therefore taken together. It is apparent from the table that in about two-thirds (137) of the cases the difference between the three occasions did not exceed 1 class interval. In a smaller number af these 137 cases the three values fell on both sides of a class border, *e.g.* in the series 7, 8 and 12 colonies, but in most cases the values fell within one and the same class. In one third of the cases the number of lactobacillus colonies recorded at two of the three examinations differed tenfold or more from one another.

In 7 of the 208 cases no lactobacillus colonies grew on any of the three occasions.

The variability of the results obtained by the tooth brush method was studied on the basis of samples removed on two occasions at an interval of 14 days from 31 patients. The differences between the first and second occasions were: less than 1 class interval in 21 cases, 1–2 class intervals in 7 cases, and more than 2 class intervals in 3 cases.

Ten patients were examined on 7 consecutive days by the swab method and six of them were regularly positive or negative, while 4 were positive on one occasion and negative on two or vice versa.

Discussion

Of the three methods studied, the spoon method proved best. An advantage of the swab method is its simplicity; a disadvantage, however, is the difficulty in stabilising the conditions and the fact that samples collected by means of a swab are liable to be less representative, for the simple reason that the swab usually becomes saturated with material from that area with which it comes into contact first. In Dubos' manual (1948, p. 707) it is to be read that sampling by means of swab is "to be avoided and discouraged as much as possible". In view of these disadvantages, the method cannot be regarded as quantitative. Sampling by means of the tooth brush method will probably give a more representative picture of the oral flora. The method is, however, troublesome. In addition, a

large volume of fluid must be used for effective rinsing of the brush, with consequent considerable dilution of the sample. This probably explains why about 60 per cent of the persons did not show any lactobacilli in samples collected by this method. Another factor partly responsible for the large proportion of lactobacillus negative patients, as judged by this method, was probably the fact that the samples were diluted with normal saline, it having been observed that a few hours' storage in normal saline was sufficient to decrease the viability of lactobacillus culture by half as compared with its original value and with a sample in which the suspension medium consisted of broth. For these reasons the tooth brush method could not be regarded as satisfactory.

Sampling and treatment of the samples by the spoon method was thus the most satisfactory. In order to obtain a representative picture of the oral flora, as much as 40 mg. of deposits on carious teeth was removed. In view of the poor oral hygiene of these mentally underdeveloped patients, collection of such a quantity offered no difficulties. It might be assumed that a fair part of the sample sometimes consisted of food rests. This was probably sometimes the case, but one might also question whether chewing of paraffin does not also remove a certain amount of food rests.

The number of lactobacilli per milligram of plaque material was assessed by STRÅLFORS (1950) as about 35. In view of this value, it was thought suitable to dilute the 40 mg. to 1 ml., because this would result in about 150 lactobacilli per 0.1 ml. solution. The selection of the dilution was fortunate, in that the results closely followed those obtained by the Hadley method (Table 1).

In the parallel investigations by the spoon method and the Hadley method, nothing suggested that the methods differed from one another in principle, because the values obtained by the two methods were of the same order in 94 per cent of the cases. Table 1, page 150) and as a greater number of colonies was found just as often by one method as by the other.

Attempts were made to compare the results obtained by the spoon method and the Hadley method with the fermentation test according to

SNYDER (1940) using both saliva and material obtained from samples collected by a Ward's carver. In our investigation, however, the Snyder method gave inconsistent results, possibly because of the essentially subjective evaluation of the colour changes. Examination of salivary samples from 41 patients on 2 different occasions by the fermentation test showed that 23 of the 41 pairs of values did not agree (48 hours reading) but were positive on one occasion and negative on the other.

As to the variability, the investigations performed showed:

1) that the variation in the weight of the sample taken in the spoon was ±10 per cent;
2) that the variation in the number of lactobacillus colonies that had grown out on different days from a lactobacillus culture dilution of given density was ±20 per cent;
3) that samples that had been taken on different days from one and the same patient under unchanged experimental conditions not infrequently differed considerably from one another, in one-third of the cases the extreme values of the three determinations differing from one another more than tenfold (Table 3).

This latter considerable variability, observed also by other investigators (HADLEY 1933, SNYDER 1940, PERMAR *et al.* 1946, and others), cannot be satisfactorily explained as being due simply to purely methodologic variations — *e.g.* variation in the amount of the primary sample, variation in the amount of inoculum, variation in the composition of the media — because the total effect of these factors was much less (see points 1 and 2 above). Neither can it be explained by the assumption that the majority of the lactobacilli were removed on the first occasion, because the intervals between the examinations were 10–14 days and, secondly, the number of lactobacilli was often highest on the second or third occasion. Possible explanations of this variability are:

that it was not possible always to remove an equal number of lactobacilli from the dental surfaces (see HADLEY 1933, and others);

that it is not possible from saliva or dental deposits to obtain a suspension in which the lactobacilli are present in uniformly

equal aggregates (see American Publ. Health Ass. 1941, PERMAR *et al.* 1946, and others);

and that there is a true variation in one and the same patient from one day to another.

Although the range of variation can be wide, it should be underlined that, as a rule, it is not difficult to determine the level of the number of lactobacilli in the mouth of an individual, because it was observed that two-thirds of the persons showed values lying within 1 class interval. It might also be mentioned that in two out of three of the 208 cases at least 2 of the 3 values fell within one and the same class interval.

Summary

In a comparison of different methods for collecting material for studying the number of lactobacilli in the oral cavity, the following observations were made:

Sampling by the use of a sterile swab and sampling by means of a tooth brush were found to be unsatisfactory. A method of collecting samples of dental deposits by means of Ward's carver and measurement of about 40 mg. of the dental deposits in a spoon, the "spoon" method, was devised and is described in detail.

Good agreement was found between the results of parallel examinations of 127 pairs of specimens collected by Hadley's (1933) method and by the "spoon" method described.

The variability in the results obtained by the use of the spoon method was studied and is described.

References

American Publ. Health Ass.: Standard methods for the examination of dairy products. New York, 1941.

APPLETON, J. L. T.: Bacterial infection in dental practice, Lea and Febiger, Philadelphia, 1944.

BÜRGER, T. J. & KALIES, W.: Trinkwasserentseuchung durch Jod, Münch. Med. Wchnschr. 21: 248, 1944.

DEWAR, M. R. & PARFITT, G. J.: Research note on the preparation of tomato peptone agar medium. Brit. Dent. Journal. XC: 150, 1951.

DIAMOND, B. E.: A selective medium for lactobacilli counts from saliva. J. Dent. Res. 29: 8, 1950.

DUBOS, R. J.: Bacterial and mycotic infections of man, Lippincott, Philadelphia, 1948.

HADLEY, F. P.: A quantitative method for estimating *Bacillus acidophilus* in saliva. J. Dent. Res. 13: 415, 1933.

KRASSE, B. & MÖLLER, O.: Ett selektivt substrat för lactobacillodling. Efterprövning av en nyligen publicerad metod. Sv. Tandl. Tidskr. 44: 133, 1951.

PERMAR, D., KITCHIN, P. C. & ROBINSON, H. B. G.: Variations in counts of lactobacilli. Made from single specimens of saliva. J. Dent. Res. 25: 475, 1946.

SNYDER, M. L. A.: A simple colorimetric method for the estimation of relative numbers of lactobacilli in saliva. J. Dent. Res. 19: 349, 1940.

SNYDER, T. L.: The relative errors of bacteriological plate count methods. J. Bact. 54: 641, 1947.

STRÅLFORS, A.: Investigations into the bacterial chemistry of dental plaques. Odont. Tidskr. 58: 151, 1950.