Survival of Human Dental Plaque Flora in Various Transport Media

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Dental plaque samples from (i) subjects with no apparent oral disease, (ii) mentally retarded subjects with periodontal disease, and (iii) subjects with active caries were collected in three transport media viz. a dithiothreitol poised balanced mineral salt solution designated as reduced transport fluid (RTF), VMG II, and modified Stuart medium (SBL). The samples were dispersed by sonic treatment, diluted in the respective medium in which they were collected, and cultured on MM10 sucrose agar. The efficiency of the transport media in the survival of dental plaque flora was determined by comparing the quantitative recovery (expressed as percentage of the initial viable count) from the specimens stored for various lengths of time. The data showed a great variation in the recovery of the oral bacterial flora from the plaque samples. VMG II and SBL served better than RTF as storage media for non-disease-associated dental plaque cultured under strict anaerobic conditions. Recoveries of bacteria from periodontal plaque specimens stored in RTF were higher than SBL and VMG II under identical conditions. The organisms present in the various plaque samples appeared to survive much better in RTF and VMG II than in SBL as determined by conventional anaerobic culturing technique. However, VMG II showed a higher recovery of organisms from these specimens with an increase in the storage period, suggesting multiplication of the plaque flora. RTF did not allow the growth of oral bacterial flora under all experimental conditions. On the basis of the relative performance of these media it is suggested that RTF is a satisfactory medium for the transport of oral bacteria present in the samples.

Human oral flora contains a large proportion of anaerobic bacteria (2, 8, 10, 11, 22; W. J. Loesche, R. N. Hockett, and S. A. Syed, Arch. Oral Biol., in press) which differ markedly in their sensitivity to oxygen (16). Although considerable efforts have been made in recent years to improve and evaluate the various techniques (2, 7, 11, 12, 15, 18, 23), little has been done to determine the efficacy of the existing transport media in maintaining the viability of the oral bacteria in the sample. Consequently, limited information is available in the literature as to the relative performance of the transport media in the survival of these organisms. Möller (19) introduced a transport medium (VMG II) which he considered superior for demonstrating streptococci and anaerobic nonsporulating bacteria in the endodontic sample. Jordan et al. (13) used VMG II medium in their studies, in which caries-inducing streptococci were isolated from human dental plaque. These investigators showed that VMG II medium retained the reproductive capacity of the organisms present in samples of dental plaque and preserved the viability of most plaque streptococci for several days at room temperature. Gästrin et al. (9) investigated the effect of storage of various clinically important pathogenic bacteria in VMG IV medium, Stuart medium as modified by Ringertz (21), and SBL medium. They found a marked decrease, i.e., 1 to 2 log, in the recovery of bacteria after their storage for 1 hr in the test media. VMG IV medium allowed multiplication of most bacteria examined by these investigators. On the basis of the comparison of the three transport media, Gästrin et al. recommended SBL medium as a suitable transport medium in different types of bacteriological examinations.

In the present study, a balanced mineral salt solution (4) poised with dithiothreitol (DTT), and containing sodium ethylenediaminetetraacetate (EDTA) was evaluated for its use-
fulness as a transport medium for various oral bacterial fluid specimens. The efficiency of this transport medium, designated as reduced transport fluid (RTF), was compared with that of VMG II and SBL media in maintaining the viability of bacterial flora present in the samples. Results of the experiments with RTF and the other two transport media are reported in this paper.

**MATERIALS AND METHODS**

**Transport media.** SBL medium was prepared according to the recommendation of Gästrin et al. except that 0.08% agar was added. VMG II medium was prepared as described by Jordan et al. (19). RTF was prepared according to the formula shown in Table 1. This medium was filter-sterilized by using a membrane filter (0.22-μm pore size). Prior to its use, each medium was tested for sterility under aerobic and anaerobic conditions. For the collection of samples, all transport media were dispensed in 10-ml samples in 18 by 150 mm test tubes. These media were also used as diluents and therefore dispensed in 9-ml amounts in 16 by 125 mm screw-cap culture tubes.

**Plating medium.** A modification of the M 10 medium of Caldwell and Bryant (4) was used as a nonselective medium for the samples. When 5% sucrose is added to this medium, polysaccharide-forming colonies of bacteria such as *Streptococcus mutans, Streptococcus sanguis*, and *Streptococcus salivarius* are readily recognized. The constituents of this medium, referred to as MM10 sucrose agar, are shown in Table 2.

**Anaerobic glove box.** An anaerobic glove box similar to the one described by Aranki et al. (2) was used for culturing of the non-disease-associated (normal) and periodontal plaque specimens under strict anaerobic conditions and for anaerobic incubation of plaques samples.

**Table 1. Composition of reduced transport fluid (RTF)**

| Components                        | Amount (ml) |
|-----------------------------------|-------------|
| 1. Stock mineral salt solution no. 1* | 75          |
| 2. Stock mineral salt solution no. 2* | 75          |
| 3. 0.1 mM EDTA                     | 10          |
| 4. 8% Na2CO3                       | 5           |
| 5. 1% dithiothreitol (freshly prepared) | 20          |
| 6. Resazurin 0.1% (optional)       | 1           |
| 7. Distilled water                 | 814         |

*Formula per liter of RTF. Prepared by membrane filter sterilization by using membrane filter (0.22-μm pore size) and dispensed into 16 by 125 mm screw-cap tubes (dilution tubes) and 18 by 150 mm test tubes (sample collection tubes). The pH of this medium was 8 ± 0.2 without adjustment, and it decreased to 7 in 48 hr in the anaerobic glove box atmosphere (85% N2, 10% H2, 5% CO2).

*Containing 0.6% K2HPO4.

*Containing 1.2% NaCl, 1.2% (NH4)2SO4, 0.6% KH2PO4, and 0.25% MgSO4.

**Table 2. Composition of MM10 sucrose agar**

| Medium                          | Amount |
|---------------------------------|--------|
| Trypticase (BBL)                | 2 g    |
| Yeast extract (Difco)           | 0.5 g  |
| Hemin                           | 0.001 g|
| Sodium lactate (60%)            | 4 ml   |
| Sodium formate                  | 1.0 g  |
| Potassium nitrate               | 0.5 g  |
| Mineral salt solution no. 1*    | 38 ml  |
| Mineral salt solution no. 2*    | 38 ml  |
| Glucose                         | 1 g    |
| Sucrose                         | 50 g   |
| Agar (Difco)                    | 15 g   |
| Distilled water                 | 874 ml |

**Autoclave the above at 121°C for 15 min and then add**

*8% Na2CO3 solution* | 5 ml |

*0.05% menadione solution* | 1 ml |

*1% dithiothreitol solution* | 20 ml |

*Defibrinated sheep blood* | 20 ml |

*Formula per liter. Medium prepared and poured on bench top and introduced into the anaerobic glove box.

*Contains 0.6% K2HPO4.

*Contains 1.2% NaCl, 1.2% (NH4)2SO4, 0.6% KH2PO4, 0.12% CaCl2.

*Sterilized by using membrane filter (0.22-μm pore size) and added to the sterile medium, cooled to 50°C. Final pH, 7.2 ± 0.2.

Inoculated plates. It contained a mixture of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide, which was circulated over a palladium catalyst to eliminate trace oxygen. The temperature of the glove box was maintained at 37°C by using a heating element and thermostatic control. The plating medium and the transport media were rendered anaerobic by incubating them in the anaerobic glove box for 48 hr prior to their use for the collection and culturing of normal and periodontal plaque samples.

**Sample collection.** Dental plaque samples were collected from three different populations. These included (i) subjects with no active caries and no evidence of periodontal disease, (ii) patients with active caries and known to harbor *S. mutans* in their plaque, and (iii) institutionalized children with periodontal disease. The first group of individuals had four males and one female. The age of these individuals ranged from 24 to 37 years. The second group consisted of six male subjects with age between 10 to 18 years. The last group consisted of four male and one female patients. All patients belonging to this group were mentally retarded and handicapped individuals. Their age varied between 11 to 22 years. In the first two populations, the dental plaque samples were removed by means of sterile, unwaxed dental floss which was passed interproximally between either the mandibular or maxillary second premolar and first molar. Three samples were collected from each subject. The floss (approximately 1 inch [2.5 cm] in length), carrying the plaque sample, was introduced into each of the three test media. When
normal subjects were used, the dispersion and culturing process was accomplished within a few minutes, so that there was a minimal delay between collection and culturing. The samples from the dental caries patients were processed 12 to 15 hr after collection.

The samples from the institutionalized children were removed by periodontal scalers, placed in a preweighed aluminum boat, weighed, and then introduced into the sample collection tubes of various prereduced transport media. Three samples for each medium for a total of nine samples were collected from each individual. One set of samples, representing all the transport media, was cultured at 4 hr; other sets were stored at refrigeration temperature for either 1 or 2 weeks before culturing.

Sample dispersion. The dispersion of periodontal plaque and normal plaque samples was accomplished by sonically treating each of the samples for 10 sec at the highest intensity of the microtip (sonifier model W185 D, Ultrasonic Inc.). Exposure of the samples to air during dispersion was minimized by equilibrating the gas space in the tube with an oxygen-free gas mixture of 95% nitrogen and 5% carbon dioxide. A set-up similar to the one recommended by the Anaerobic Laboratory, Virginia Polytechnic Institute, (1) was used to gas the sample tubes during the entire process. The dispersed samples were closed by rubber stoppers and transferred immediately to the anaerobic glove box for dilution and plating process.

Curious plaque samples were dispersed in the same manner as other specimens except that no gas mixture was used during the process.

Sample dilution and plating procedure. The plaque samples from normal subjects were collected in prereduced transport media and handled under strict anaerobic conditions throughout the experiment. They were diluted anaerobically in the same type of medium in which they were collected and stored. Serial 10-fold dilutions were prepared, and the last three dilutions were used for plating on a prereduced medium. After the initial plating was completed, the diluted samples were kept inside the anaerobic glove box until the second plating was done 6 hr later. Then the tubes were closed by rubber stoppers, removed from the glove box, and stored at 10 C except when used for the experiment.

Periodontal plaque samples were diluted and plated in the anaerobic glove box. The procedure used was similar to the one used for normal plaque samples except that there was an initial 4-hr delay between the collection of the specimens and culturing procedure.

The samples obtained from the caries-active patients were collected in freshly prepared transport media, diluted and plated on the bench top in the room atmosphere, and incubated in the same anaerobic glove box used for other samples. Each sample was diluted in the same type of medium in which it was collected and stored.

RESULTS

The recovery of the bacteria from normal plaque specimens was followed for 14 days. The viable counts obtained from the first culturing were considered as a reference value (100%). The results are shown in Table 3. There was approximately a 19% reduction in the viable counts after 6 hr of storage of the specimens in RTF. The VMG II and SBL showed a slight increase in the recovery of the organisms, indicating possible multiplication. Approximately 70% of the organisms were destroyed after 1 day of storage in RTF as compared to 28 to 30% loss in viability in VMG II and SBL medium under identical conditions. The survival of bacteria appeared to be the highest in VMG II medium. After an initial decrease in viability, the number of bacteria remained stable in this medium during the entire storage period.

At 7 and 14 days, samples were plated from either dilutions prepared on day 0 or from new dilutions of the initial collection tubes. There was no apparent difference between the fresh or old dilutions on the recovery of organisms from the samples.

Our next experiments were concerned with field studies involving collection of plaque from periodontally involved, mentally retarded individuals. These subjects had considerable plaque accumulations on their tooth surfaces so that it was possible to collect and weigh the plaque samples. Therefore, in this manner, the data could be referred to a milligram wet weight reference. Nine samples were removed from each of five subjects and were returned to the laboratory in the respective transport media. One set of samples was dispersed and plated at 4 hr. The other sets were stored at 10 C until dispersed and plated at either 7 or 14 days. The results are given in Table 4. At all time periods, the recoveries per milligram of plaque wet weight were superior for the RTF. After 14 days, there was about a 40% decrease from the 4-hr value with RTF. However, with VMG II there was a 65% decrease and with SBL a 35% decrease.

S. mutans has been implicated as the etiologic agent of dental caries in certain animal model systems (14). Considerable efforts are currently being made to determine the presence and level of this organism in human populations. In the next series of experiments, interproximal plaque was removed from six teen-age subjects known to harbor S. mutans and S. sanguis in their plaque. As these organisms are microaerophilic, the anaerobic precautions taken earlier with other samples were omitted and the samples were dispersed, diluted, and plated on the bench top prior to incubation in the anaerobic glove box. This method of sample manipulation would more
### Table 3. Recovery of dental plaque flora of normal subjects from three transport media under strict anaerobic conditions

| Culturing in days after collection | Percent survival of bacteria | Statistical significance* |
|-----------------------------------|-----------------------------|---------------------------|
|                                   | 1 RTF | 2 VMG II | 3 SBL | 1 vs 2 | 1 vs 3 | 2 vs 3 |
| 0                                 | 100   | 100      | 100   |        |        |        |
| (14.5 ± 7.7 × 10^3)               |       | (18.6 ± 5.9 × 10^3) | (18.6 ± 12.6 × 10^3) |        |        |        |
| 1/4                               | 81.2  | 102.8    | 103.1 | <0.05  | NS     |        |
| 1                                 | 30.6  | 72.4     | 70.3  | <0.01  | <0.005 | NS     |
| 2                                 | 23    | 73.7     | 62.8  | <0.001 | <0.005 | NS     |
| 4                                 | 5.5   | 78       | 28.9  | <0.001 | <0.01  | <0.025 |
| 7                                 | 4.0   | 78.3     | 21.7  | <0.001 | <0.001 | <0.001 |
| 14                                | 3.8   | 78.4     | 16.4  | <0.001 | <0.001 | <0.001 |

* Floss samples were cultured in the anaerobic glove box and stored anaerobically at 10 C.

* Based on t-test (3).

Numbers in parentheses: mean counts of five samples on pre-reduced MM10 sucrose agar, plus or minus standard deviation. These counts are given as a reference value.

* Not significant.

### Table 4. Recovery of dental plaque flora of periodontal patients from three transport media under different experimental conditions

| Sample storage in days | Viable counts x 10^4/mg wet weight | Statistical significance* |
|------------------------|-----------------------------------|---------------------------|
|                        | 1 RTF | 2 VMG II | 3 SBL | 1 vs 2 | 1 vs 3 | 2 vs 3 |
| 1/4                   | 3.9 ± 0.9 | 2.08 ± 0.9 | 2.3 ± 0.2 | <0.025 | <0.01  | NS     |
| 7                     | 2.3 ± 2.8 | 0.9 ± 0.7 | 1.0 ± 1.0 | NS     | NS     | NS     |
| 14                    | 2.4 ± 2.8 | 0.95 ± 0.9 | 1.5 ± 1.9 | NS     | NS     | NS     |

* Samples were stored undispersed in the transport media at 10 C and sonically treated on the day they were used for culturing.

* Based on t-test (3).

* Represents mean counts of five plaque samples for each transport medium, plus or minus standard deviation.

* Not significant.

closely approximate the conditions for handling similar samples in epidemiologic surveys. There was a 12- to 15-hr delay between collection and the first culturing of the samples. The samples after dispersion were stored at 10 C and cultured over a 14-day period to determine the relative performance of the transport media in the survival of the plaque flora. The results (Table 5) indicate that the recovery of plaque bacteria from SBL transport medium was very poor. There was almost 100% loss of the viability of the organisms in this medium. The recovery of the plaque bacteria from RTF and VMG II within the first week dropped to 23% and 33%, respectively. In the second week of storage, the recovery of organisms from VMG II medium was increased tremendously, suggesting multiplication of bacteria or yeast, or both.

Differential counts of S. mutans and S. sanguis were made on MM10 sucrose agar. These organisms form characteristic colonies due to polysaccharide formation which can be easily identified. In addition, certain key tests were performed on the representative and doubtful isolates to confirm their identification. These included mannitol fermentation, ability to form ethanol-insoluble dextran, and heavy plaque formation on wire (17). The recovery of these organisms from the transport media was followed for 14 days. The results of this study are presented in Table 6. There was a rapid decrease in the viability of S. mutans and S. sanguis in SBL medium as compared to the other transport media. There was a 75 to 90% decrease in the viability of the organisms in RTF medium over a 10-day period. S. mutans appeared to survive best in VMG II. However, this medium promoted growth of S. sanguis as shown in Table 6.

### DISCUSSION

The problem of delay between the collection of the specimens and subsequent culturing is
Table 5. Survival of dental plaque flora of dental caries patients in three transport media cultured at different time periods

| Culturing in days | Percent survival of bacteria | Statistical significance* |
|-------------------|------------------------------|--------------------------|
|                   | 1 RTF                        | 2 VMG II                 | 3 SBL         | 1 vs 2 | 1 vs 3 | 2 vs 3 |
| 1/2               | 100 (16.2 ± 8.0 × 10⁴)        | 100 (19.0 ± 13.0 × 10⁴)  | 100 (10.1 ± 5.0 × 10⁴) | NS*    | <0.01  | <0.005 |
| 4                 | 32.2                         | 47.3                     | 13.2          | NS*    | <0.001 | <0.001 |
| 7                 | 23.0                         | 32.9                     | 1.7           | NS*    | <0.005 | <0.005 |
| 10                | 16.7                         | 98.8                     | 0.1           | <0.005 |        |        |
| 14                | 2.4                          | 96.7                     | 0             | <0.005 |        |        |

* Samples were handled aerobically and stored at 10 C. The inoculated MM10 sucrose agar plates were incubated in the anaerobic glove box.

* Based on t test (3).

* Numbers in parentheses: mean counts of six floss samples, plus or minus standard deviation. These counts are given as a reference value.

* Not significant.

Table 6. Effect of storage on the recovery of Streptococcus mutans and S. sanguis from three transport media

| Culturing in days | Percent survival of S. mutans and S. sanguis |
|-------------------|---------------------------------------------|
|                   | S. mutans                                     | S. sanguis                           |
|                   | RTF                                          | VMG II                               | SBL                |
| 1/2               | 100 (3.7 ± 4.7)*                             | 100 (20.3 ± 16.6)                    | 100 (28.5 ± 63)    |
|                   | (9.5 ± 10.6)                                 | (7.7 ± 2.9)                          | (6.75 ± 5.1) |
| 4                 | 34.9                                         | 80.0                                 | 74.3               |
| 7                 | 12.0                                         | 77.2                                 | 88.4               | 5.4   | 9.12 |
| 10                | 10.3                                         | 20.5                                 | 205.0              | 0.65  | 0.14 |
| 14                | 0.3                                          | 2.5                                  | 185.0              | 0     | 0    |

* Results are based on the mean of six carious plaque samples collected in the transport media from patients known to harbor S. mutans. Viable counts were made on MM10 sucrose agar.

* Numbers in parentheses: mean viable counts × 10⁴ per dental floss, plus or minus standard deviation. These counts are given as a reference value.

not uncommon. Ideally, our aim was to select a reasonably good medium which could maintain the viability of the overall population of the organisms present in the plaque without appreciably altering their relative proportion over a period of time. VMG II and SBL media were selected as they are routinely used for the transport of various specimens for bacterial analyses. RTF is currently being used as a transport medium in our laboratory. Therefore, the performance of this medium was compared with other two media under various conditions.

Culturing of the normal plaque samples under continuous anaerobiosis throughout the entire experimental period showed that SBL and VMG II medium served better as storage media than RTF. The viability of the plaque bacteria was well maintained in these media for several days under these conditions. However, these ideal conditions would not be duplicated in the usual circumstances under which plaque is collected and cultured.

When the samples were collected from periodontal patients under field conditions and manipulated and plated anaerobically, the RTF yielded the highest recoveries on a milligram (wet weight) basis at all time intervals. This would suggest that for plaque associated with periodontal disease the RTF might be the transport medium of choice. This performance may be attributed to the low Eₜ of the RTF which could maintain the viability of the anaerobes which tend to be present in high proportions in periodontal plaque (Loesche et al., Arch. Oral Biol., in press).

The results obtained from the cultural study of carious plaque samples showed that SBL medium did not serve well as a storage medium. The RTF and VMG II were comparable.
for the first 7 days, but, with further storage, VMG II gave rise to higher recoveries. A careful analysis of the composition of the VMG II provides the basis of its apparently excellent performance. VMG II medium contains 1.1% peptones (gelatin, thiotone, and trypotor) and 1% glycerophosphate, which may serve as growth-promoting substances. Möller (19) used small amounts of phenyl mercuric acetate (0.093%) as a bacteriostatic agent in this medium apparently to keep growth in check. However, the presence of SH-containing compounds, such as thioglycollic acid (0.05%) and cysteine (0.05%), could neutralize the bacteriostatic effect of the mercurials in the medium (21), thereby allowing growth to occur.

Gastriën et al. (9) found that most of the pure cultures of pathogenic organisms included in their study showed growth and multiplication in VMG IV medium. Jordan et al. (13), working with dental plaque samples, also observed the same phenomenon and stated that the reproductive capacity of the bacterial cells was well maintained in VMG II medium. Rundell et al. (B. B. Rundell, L. A. Thomson, W. J. Loesche, and H. M. Stiles, Abstr. International Association for Dental Research, no. 576, 1972) studied the survival of S. mutans, S. sanguis, S. mitis, and S. salivarius in VMG II, SBL, and RTF. They reported that growth sometimes occurred in VMG II medium as determined by total viable counts of the organisms. SBL medium was found to be the most toxic of the three transport media tested by these investigators. RTF, on the other hand, gave rise to the most constant population of oral streptococci under these conditions.

In our storage studies, we found the growth-promoting ability of VMG II medium (Table 5) when the samples were dispersed under aerobic conditions. Higher recovery of S. sanguis over an increase storage period of the samples (Table 6) would mean its multiplication in VMG II medium. These findings suggest that the facultative or microaerophilic bacteria, or both, could multiply in this medium. Further studies would be required to determine the qualitative nature of specific bacterial components of the plaque flora which could grow and metabolize the nutrients present in this medium. Due to these limitations, VMG II should be used with caution as a transport or storage medium, especially when one is interested in determining the relative proportion of the organisms in the original samples.

The present findings indicate that the ideal medium for the storage and transport of all the different types of bacterial specimens is not yet available. However, of the three media tested, RTF would appear to be more satisfactory for dental samples. It is a nonselective transport and storage medium. With the exception of 0.02% DTT which was added to the medium as a reducing agent, it does not have any compound which could support the growth of bacteria. DTT was selected as a reducing agent because of its low oxidation-reduction potential and minimal susceptibility to oxidation under aerobic conditions (6). The addition of very low concentrations of EDTA (0.001 M) to RTF appears to promote a more uniform dispersion of bacterial cells (Loesche et al., Arch. Oral Biol., in press). Since EDTA acts as a chelating agent, its incorporation into RTF was useful for binding divalent cations, such as Ca++, which are present in high concentration in plaque and contribute to the aggregation of plaque bacteria.

RTF does not contain any bacteriostatic agent that would affect the multiplication of bacteria in the sample. Consequently, growth might occur in this medium if the sample itself contains enough nutrients to allow bacterial multiplication for a limited period. However, our studies did not show any growth of the plaque organisms in this medium. Although its efficiency as a storage medium varied with the nature of the samples, the performance of RTF appears to be better than the other two media tested. Posing the medium with DTT makes it more resistant to oxidation under aerobic conditions. This characteristic of RTF may reflect its better performance in the recovery of the plaque flora from carious and periodontal plaque samples. These findings suggest that RTF could be used as a desirable medium for transport and storage of oral specimens. The viability of the organisms can be best maintained in this medium at refrigeration temperature (Rundell et al., Abstr. International Association for Dental Research, no. 576, 1972).

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