Preservation and Transportation of Bacteria by a Simple Gelatin Disk Method

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Received 1 October 1980/Accepted 16 March 1981

A wide range of bacterial species, e.g., Enterobacteriaceae and Neisseria, Streptococcus, Branhamella, Haemophilus, Gemella, Pseudomonas, Flavobacterium, and Bacteroides species, were successfully preserved for 1 to 5 years by our gelatin disk drying method. The β-lactamase activity of penicillinase-producing Neisseria gonorrhoeae was retained for more than 3 years with this method. Good results were also obtained upon airmailing many strains of N. gonorrhoeae embedded in gelatin disks from Japan to the United States. Neisseria, Branhamella, Gemella, and Haemophilus organisms suspended in the reagent used in the preparation of the gelatin disks could be preserved for 6 to 12 months after freezing the cell suspensions at −20°C. Furthermore, modification of our gelatin disk preservation method made possible the safe long- and short-distance transportation of clinical isolates. Our methods can be used by any small laboratory, since they require only conventional instruments and reagents.

The long-term preservation of bacteria, accompanied by the stable maintenance of their properties, is important for any laboratory. To this end, the lyophilization (freeze-drying) method has been widely used since Flosdorf and Mudd (6, 7) established basic experimental conditions. However, due to the cost of the necessary instruments and the complexity of the lyophilization procedures, this method cannot always be used in small laboratories.

By modifying the method of Stamp (17), we developed a gelatin disk method which made possible the successful long-term preservation of Neisseria gonorrhoeae (20). We now report that this method is also applicable in the preservation of several other bacterial species.

Furthermore, the same media and procedures used in the preparation of the gelatin disks are applicable for the domestic and international transportation of bacterial strains, including N. gonorrhoeae strains.

MATERIALS AND METHODS

Strains. (i) Preservation study. A total of 318 strains, consisting of 15 genera and 42 species of bacteria, were used. The bacterial species or groups, numbers of strains, survival periods, and isolation sources are listed in Table 1.

(ii) Transportation study. For international transportation in gelatin disks, 108 stock strains of N. gonorrhoeae, consisting of 7 penicillinase-producing strains (PPNG) (1, 16), 1 fresh isolate highly suscep-
tible to penicillin (control) (Table 2), and 100 other strains (Table 3), were used.

For domestic transportation of fresh isolates, 2 strains of Neisseria meningitidis from cerebrospinal fluid, 1 isolate of Neisseria mucosa from pleural exudate, and 112 strains of viridans group streptococci, including six species, derived from periodontitis specimens were used.

Culture media and methods. Agar culture plates were used for growing, collecting, and preserving bacterial colonies or for confirmation of cell survival: GCB-2DS medium (9) was used for Neisseria and Branhamella, GCBH medium (9) was used for Flavobacterium and Gemella, heart infusion agar (Difco) with 4% defibrinated horse blood was used for Streptococcus, and Trypticase soy agar (BBL) with 5% Fildes enrichment (Difco) was used for Haemophilus. These organisms were incubated at 35°C in a candle extinction jar. Vibrio paraheamolyticus was cultured aerobically at 35°C, using brain heart infusion agar (BBL) containing 2.5% NaCl, 0.001% crystal violet (Sigma), and a 5% human erythrocyte suspension (12). Bacteroides was inoculated onto Gifu anaerobic medium (GAM Agar, Nissui) and incubated anaerobi-
cally, using an ANAERO-BOX AZ (Hirasawa Works). The other bacteria were aerobically grown at 35°C on heart infusion agar (Difco).

Species identification. Isolates of Neisseria and Branhamella were identified according to the method of Catlin (3). Viridans streptococci, V. paraheamolyticus, Haemophilus, Staphylococcus, Flavobacterium, and Bacteroides isolates were classified according to the methods of Facklam (5), Miyamoto et al. (12), Young (21), Ivler (8), Tatum et al. (19), and Sutter et
Table 1. Bacteria preserved by the gelatin disk method

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains</th>
<th>Storage yr</th>
<th>Survival period (yr)</th>
<th>Note or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>131</td>
<td>1976</td>
<td>4</td>
<td>Urethra, cervix</td>
</tr>
<tr>
<td>PPNG</td>
<td>2</td>
<td>1977</td>
<td>3</td>
<td>CDC reference strain</td>
</tr>
<tr>
<td>Neisseria meningitidis</td>
<td>16</td>
<td>1976</td>
<td>4</td>
<td>Cerebrospinal fluid, throat</td>
</tr>
<tr>
<td>Neisseria lactamica</td>
<td>12</td>
<td>1977</td>
<td>3</td>
<td>Throat</td>
</tr>
<tr>
<td>Neisseria mucosa</td>
<td>1</td>
<td>1977</td>
<td>3</td>
<td>Throat</td>
</tr>
<tr>
<td>Neisseria subflava</td>
<td>1</td>
<td>1977</td>
<td>3</td>
<td>Throat</td>
</tr>
<tr>
<td>Neisseria flavescens</td>
<td>1</td>
<td>1977</td>
<td>3</td>
<td>Throat</td>
</tr>
<tr>
<td>Branhamella catarrhalis</td>
<td>1</td>
<td>1976</td>
<td>4</td>
<td>Throat</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>1</td>
<td>1976</td>
<td>4</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>Streptococcus groups A, A', B, C, D, E, F, G, K, L, N, Q, and MG*</td>
<td>15</td>
<td>1977</td>
<td>3</td>
<td>Standard strains for serogrouping</td>
</tr>
</tbody>
</table>

* Strains examined were J17A4 (A), PHLS (A'), 090 R and B Lewis (B), Chestle (C), NCTC 8307 and PZH D3 (D), K131 (E), O'Mahoney (F), Valente (G), M. Cuca "Charwoman" (K), SIC 16 (L), J. O. Lactis (N), E844 (Q), and ScMG-8037 NCTC (MG).

** S. sanguis I, 6 strains; S. sanguis II, 81 strains; S. mitis, 18 strains; S. MG-intermedius, 2 strains; S. anginosus-constellatus, 4 strains; and S. morbillorum, 1 strain (5).

al. (18), respectively. The identifications of Enterobacteriaceae, Pseudomonas, and Gemella isolates were in accordance with Cowan and Steel's Manual for the Identification of Medical Bacteria (4). The chromogenic cephalosporin method (14) was used to examine the β-lactamase of PPNG.

Preparation of gelatin disks and strain preservation. The procedures for the preparation of our gelatin disks have been reported previously (20). However, in view of practice, some additional comments are in order.

Reagent A consists of soluble 5% dextrose (Difco) and 3% skim milk (Difco) in distilled water suspended with 0.6% decoloring carbon (Norit Extra, NV Norit-Vereinigung) and is autoclaved at 110°C for 10 min. Thereafter, this suspension can be preserved in a refrigerator for a long period. Although the carbon powder is not always necessary, its addition is recommended when the strain is vulnerable to impairment by toxic substances in the culture medium, as is the case for Neisseria gonorrhoeae (11, 22). The pH of this reagent is slightly acidic; therefore, if a slightly alkaline reaction is desired, its pH can be adjusted to 7.2 to 7.6 by adding 1 N NaOH solution. For reagent B, a 5% sodium L-ascorbate (Wako Co.)-distilled water solution is prepared and sterilized by filtration just before use. Alternatively, the filtrate can be safely preserved for several weeks if, immediately after filtration, small amounts of the filtrate are dispensed, sealed, light-shielded, and frozen at -20°C or below. After thawing, the unused reagent should be discarded. Reagent C is a 20% gelatin (Difco)-distilled water solution which can be preserved for a long time if it is refrigerated after it has been heat dissolved and autoclaved at 121°C for 15 min.

After the initial drying process, the viable cell count of Neisseria gonorrhoeae decreases by 10^{-2} (20); those of other bacteria decreased by 10^{-1} (17). Therefore, to compensate for the expected decline in the viable cell count, very dense cell suspensions, i.e., 10^8 to 10^{10}/ml, must be prepared. For storage, cells in the logarithmic growth phase, and not those in the stationary phase, should be collected.

The suspension reagent was prepared by mixing reagents A, B, and C at a volume ratio of 1:0.2:1. To obtain 2.2-ml cell suspensions, one or two plates were used for the collection of colonies from strains exhibiting vigorous growth, e.g., strains of Enterobacteriaceae; for the collection of strains manifesting smaller colonies, e.g., pathogenic Neisseria or Streptococcus strains, at least three plates were used. Proliferated cells were collected with a bacteriological loop and
inoculated into the suspension reagent, which had been warmed to 35°C. The cell suspension was then thoroughly mixed with a vibrator to obtain homogeneity and inoculated with a sterile capillary pipette onto paraffin-soaked filter papers in petri dishes. Inoculation was in dropwise fashion at appropriate intervals, to form concentric circles. The 2.2-ml cell suspension could be divided into approximately 60 drops (ca. 0.03 ml per drop). If it was immediately sealed and frozen at -20°C, the unused portion of the cell suspension could be used at a later date. The droplet-bearing filter paper in the petri dishes was then placed into a glass desiccator with 500 g of silica gel; a wide-mouthed glass cylinder containing 200 to 300 g of phosphorus pentoxide (P_2O_5) was also put into the desiccator. To facilitate drying, two glass rods were horizontally inserted between the top and bottom of each pair of stacked petri dishes. The desiccator was closed, connected via an intermediate cotton-filter-carrying tube to a vacuum pump, and evacuated until a reduced pressure of ca. 20 mmHg (ca. 2.7 kPa) was reached. Then the desiccator was left to stand at room temperature for 4 to 7 h and drained, and the wet surface layer of P_2O_5 was removed carefully so as to avoid skin contact. The water-soaked P_2O_5 layer was discarded by admixing small amounts with large amounts of tap water in a bottle inside an air-sucking chamber. The desiccator was then evacuated to 10 mmHg (ca. 1.3 kPa) or below, closed, and left to stand overnight at room temperature. When these procedures were followed, the droplets were completely dry by the next day and formed circular thin disks attaching to the filter paper. The disks were lifted from the paper with sterile forceps and placed into 5-ml glass vials containing silica gel and cotton and filter paper separators. These vials were then tightly sealed and stored at -20°C.

Before storage, one of the disks from each lot was redissolved in ca. 0.1 ml of heart infusion broth (Difco) or sterile distilled water, and the bacterial suspension was briefly warmed to 35°C and stirred, using a vibrator. This was followed by inoculation onto a culture plate to confirm the expected cell viability count and the absence of contamination.

Neisseria, Branhamella, Haemophilus, and Gemella strains were used in a modification of the preservation technique in which desiccation was omitted. A 0.1-ml suspension of each of these strains was inoculated into a small stoppered glass tube and stored at -20°C.

Transportation of strains preserved by the gelatin disk or the modified method. Seven strains of PPNG and a penicillin-susceptible control strain of N. gonorrhoeae were subcultured after redissolving stock gelatin disks. Immediately before dispatch, gelatin disks were prepared with these new cultures and three to five disks per strain were placed into a 2-ml A/B NUNK (Denmark) serum tube (N-1076-1) which was sealed and airmailed to the Centers for Disease Control (CDC), Atlanta, Ga. (Table 2).

Similarly, 100 stock strains of N. gonorrhoeae (patient isolates) were airmailed to J. S. Knapp at the Neisseria Reference Laboratory, Seattle, Wash., for auxotyping. Before dispatch, these strains were also checked for viability, using one of the disks from the same lot (Table 3).

To collect isolates from patients domestically and to test their preservation in the gelatin disks, the following procedures were applied. Cell-free gelatin disks, prepared as described above, were placed into glass tubes (6 by 60 mm; one disk per tube). These were closed with a stopper, sealed, and surface mailed to the pertinent institutions, where they were dissolved in 0.05 to 0.1 ml of sterile distilled water (37°C). One loopful of proliferated cells from a pure culture derived from the specimen was inoculated into this solution, which was stirred thoroughly with a vibrator to obtain a homogeneous suspension. Thereafter, the drying procedures were as described above, and the disks bearing the bacteria, in small sealed glass tubes, were returned to our laboratory by surface mail.

Since in this method no special care need be taken to prevent bubble formation and consequent disk deformation, desiccation at a pressure of 10 mmHg or below can be applied from the beginning, to attain complete desiccation overnight. Furthermore, silica gel, instead of P_2O_5, may be used as the desiccant.

RESULTS

Preservation study. Preservation was checked in 1980. Cell survival was evaluated by culturing one disk from the same lot of each strain.

Flavobacterium meningosepticum, viridans streptococci, and Gemella haemolysans had been stored relatively recently; therefore, the period of survival for these organisms was a reflection of the recent storage rather than the applicability of the preservation method (Table 1). All other tested bacterial species survived 3 to 5 years. All N. gonorrhoeae and N. meningitidis strains survived for at least 4 years (see Table 3), whereas some strains of N. gonorrhoeae manifested a remarkable decrease in viability count after 3 years of preservation. The two tested PPNG strains not only survived for 3 years but also retained their β-lactamase activity. In the case of strains with strong gelatinase activity, e.g., Pseudomonas aeruginosa strains, the viscosity of the suspension reagent containing the large number of the organisms was decreased. However, this partial loss of viscosity could be avoided by decreasing the size of the inoculum to 10^6 cells per ml without impairing cell survival.

We were unable to successfully apply our gelatin disk method in the preservation of Vibrio cholerae strains. No cell proliferation was discernible at 1 week postpreservation at -20°C, although immediately after desiccation the viability count had been satisfactory.

Our modified nondesiccate preservation technique provided highly satisfactory results. N. gonorrhoeae survived for 6 months. N. menin-
gitidis, other species of Neisseria, Haemophilus influenzae and G. haemolysans survived for 1 year. These species could tolerate and survive two to three freezings and thawings.

**Transportation study. (i) International.** Seven PPNG strains and a susceptible control strain airmailed from Japan to the CDC were exposed to the climatic conditions of three seasons (Table 2). The CDC confirmed that all of these strains showed abundant growth. In addition, examination of β-lactamase activity revealed that the pre- and postmailing evaluations were similar. N. gonorrhoeae strains embedded in our gelatin disks no later than 1976 were airmailed at the end of 1979 to Seattle, Wash., for auxotyping. The survival rate of these strains was 76.1% (35/46); all 54 strains embedded in or after 1977 survived (Table 3). Accordingly, the overall survival rate of these 100 N. gonorrhoeae strains was 89%.

(ii) **Domestic.** In the domestic transportation study, the disks bearing bacteria were in transit for 3 to 4 days. All the examined strains were safely recovered in our laboratory.

**DISCUSSION**

The maintenance and preservation of bacterial strains is a matter of importance even for small laboratories, and the costs of special instruments, manpower shortages and the space required for the storage of increasing numbers of stock strains represent real problems. Therefore, we searched for an alternative method to the successive transfer or lyophilization techniques.

Stamp (17), who explored a variety of factors affecting bacterial survival upon drying, reported a simple preservation method which involves using gelatin as the suspension medium and in vacuo drying of the organisms at room temperature. Although his method was successful in the long-term preservation of bacterial species of the Enterobacteriaceae and of the genera Chromobacterium, Pasteurella, Erysipelothrix, and Streptococcus, it failed in the prolonged preservation of N. meningitidis and V. cholerae.

Our modification of Stamp’s method resulted in the successful preservation of N. gonorrhoeae (20), including stable maintenance of colony forms and of levels of antibiotic susceptibility even after 3 years of preservation. In the present investigation we increased the number of examined strains and species of the genus Neisseria and included 14 other bacterial genera (Table 1). All examined bacteria, except those which had been placed in storage relatively recently, were found to survive for 3 to 5 years. Although V. cholerae could not be successfully preserved by our method, V. paraaemolyliticus survived for 4 years. Furthermore, the β-lactamase activity of PPNG remained at the preembedding level even after 3 years of storage.

We attribute our successful preservation of Neisseria strains to the rapid drying by strong desiccation at 20 to 10 mmHg. Our improving on the constituents which make up the suspension medium may also have contributed to our satisfactory results.

In an independent experiment, we used Stamp’s original drying method, which involves a drying period of 2 to 3 days and a pressure of 100 to 300 mmHg (ca. 13 to 27 kPa). We found that no N. gonorrhoeae strains survived this procedure. Based on our present findings, we suggest that our preservation method is applicable to Haemophilus and other bacteria which are vulnerable to artificial media.

We found that Neisseria and other fastidious bacterial cells suspended in the same reagent as was used in the preservation of the gelatin disks survived for 6 to 12 months upon freezing at

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Allotted no.</th>
<th>Date</th>
<th>Results</th>
<th>( \beta )-Lactamase</th>
</tr>
</thead>
<tbody>
<tr>
<td>52-45</td>
<td>79-030555</td>
<td>11 November 1978</td>
<td>19 December 1978</td>
<td>+</td>
</tr>
<tr>
<td>53-63</td>
<td>79-030556</td>
<td>11 November 1978</td>
<td>19 December 1978</td>
<td>–</td>
</tr>
<tr>
<td>54-30</td>
<td>79-106990</td>
<td>2 August 1979</td>
<td>20 August 1979</td>
<td>+</td>
</tr>
<tr>
<td>54-40</td>
<td>79-106991</td>
<td>2 August 1979</td>
<td>20 August 1979</td>
<td>+</td>
</tr>
<tr>
<td>54-49</td>
<td>80-024289</td>
<td>14 November 1979</td>
<td>3 December 1979</td>
<td>+</td>
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<tr>
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<tr>
<td>54-64</td>
<td>80-059722</td>
<td>10 March 1980</td>
<td>25 March 1980</td>
<td>+</td>
</tr>
<tr>
<td>55-01</td>
<td>80-059723</td>
<td>10 March 1980</td>
<td>25 March 1980</td>
<td>+</td>
</tr>
</tbody>
</table>

* Gelatin disks were prepared immediately before dispatch and transported at room temperature. Numbers were allotted and the results were examined at the CDC.

* \( \beta \)-Lactamase-negative, susceptible strain.
This finding facilitated the repeated examination of such fastidious organisms by dispersing the cell suspensions into small amounts and then freezing the dispersed suspensions for future studies.

The transportation of fastidious organisms also presents a problem which hampers international cooperative efforts. However, our success in sending the first PPNG isolated in Japan (15) to the United States showed that this problem could be overcome by embedding the organisms in our gelatin disks. As our preservation method is simple and does not require instruments other than those readily available in almost any laboratory, it facilitates international cooperative surveys on PPNG and other fastidious strains.

Our preservation method also made it possible to send N. gonorrhoeae isolates obtained from Japanese patients to the United States for auxotyping. At the request of J. S. Knapp, we dispatched 100 strains in our gelatin disks (two to three disks per strain) in a small airmail package to aid in the determination of whether there exists an auxotype (2) responsible for the dissemination of gonococcal infections (10). Organisms were recovered and auxotyped from all disks that had been stored for less than 3 years. Recovery was not possible from 11 strains that had been stored for more than 3 full years (Table 3), possibly due to temperature exposure during or after mailing. Studies are under way in our laboratory to determine whether stronger desiccation may improve the survival of the organisms, particularly those intended for long-distance mailing, by making them less susceptible to the effects of temperature.

Dried suspension medium or cell-free gelatin disks were also used for transporting isolates from a collection center to our reference laboratory, where all the strains were recovered and identified. Our preservation method makes it possible for most laboratories to engage in this type of work, since the required techniques are simple and the necessary instrumentation is readily available in most laboratories. The vacuum level at a pressure of 50 mmHg (ca. 6.7 kPa) can be reached by using a commercially available metal water-powered aspirator which is connected to a normal water faucet.

Much work has been done in Japan (13) on the applicability of Stamp's method (17) to the preservation of various bacterial species. Further studies may lead to the successful preservation of V. cholerae and other fastidious species by the gelatin disk method. To this end, we are investigating such factors as the required conditions of the suspension medium, the incubation periods and methods, and the temperature at which optimal preservation can be achieved.

**LITERATURE CITED**

1147–1149.