

Comparison of different culture media and storage temperatures for the long-term preservation of *Streptococcus pneumoniae* in the tropics

George Siberry,¹ K.N. Brahmadathan,² Rajeswar Pandian,³ M.K. Lalitha,⁴ Mark C. Steinhoff,⁵ & T. Jacob John⁶

Objective The preservation of *Streptococcus pneumoniae* by standard freezing methods for subsequent tests — such as serotyping and antibiotic susceptibility — is not possible or is difficult in many developing countries because of the high cost of equipment, inadequate equipment maintenance, and irregular power supply. We evaluated alternative low-cost methods, by comparing different culture media and storage temperatures.

Methods Clinical isolates of five capsular types (1, 5, 7, 19, and 23) of *S. pneumoniae* were preserved in rabbit blood, sheep blood, skimmed milk, or glycerol–chocolate broth, and stored at –20 °C or –70 °C. The cultures were also preserved by lyophilization or sand desiccation, followed by storage at room temperature and 4 °C. The viability of the preserved cultures was determined by making serial colony counts on day 0 and after 1 week, 4 weeks, 4 months and 16 months. The viability of cultures preserved by sand desiccation and storage at 4 °C was also determined every 6 months for up to 68 months.

Findings Irrespective of the media used, cultures maintained at –20 °C became nonviable by the fourth month, while those maintained at –70 °C were still viable at 16 months. Cultures preserved by lyophilization or sand desiccation lost their viability by the fourth month when maintained at local room temperature (30–42 °C), but remained viable when stored at 4 °C for up to 68 months.

Conclusions Our results confirm that freezing at –70 °C, or lyophilization and storage at 4 °C are the ideal methods for the preservation of *S. pneumoniae*. In laboratories where lyophilization is not feasible, sand desiccation and storage at 4 °C offers an alternative low-cost method for the long-term preservation of *S. pneumoniae*.

Keywords: Streptococcus pneumoniae, preservation; culture media; cryopreservation, methods; lyophilization, methods; desiccation, methods; tropical climate.

Mots clés: Streptococcus pneumoniae, isolement et conservation; milieu de culture; conservation par le froid, méthodes; lyophilisation, méthodes; dessiccation, méthodes; climat tropical.

Palabras clave: Streptococcus pneumoniae, aislamiento y purificación; medios de cultivo; criopreservación, métodos; liofilización, métodos; desecación, métodos; clima tropical.

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Introduction

Pneumococcal infections cause substantial morbidity and mortality in infants and children in many

developing countries (1, 2). Studies suggest that *Streptococcus pneumoniae*, together with *Haemophilus influenzae*, may be responsible for half of all pneumonias in children and 20–40 % of all bacterial meningitis cases in the developing world, but little is known about their serotype distribution and age-specific disease rates (3, 4). Information on the regional distribution of pneumococcal serotypes is essential for the development and use of appropriate pneumococcal vaccines in developing countries (3). A multi-centre hospital surveillance study was therefore initiated in Vellore, Tamil Nadu, India in 1993 under the India Clinical Epidemiology Network (INDIACLEN), with support from the US Agency for International Development through the International Clinical Epidemiology Network (INCLEN). The objective was to describe the epidemiology of *S. pneumoniae* invasive infections in six hospitals located in widely different areas of

¹ Fellow, Department of Paediatrics, Johns Hopkins Medical Institutions, 600 N Wolfe Street, Baltimore, MD 21287, USA (email: gsiberry@worldnet.att.net). Correspondence should be addressed to this author.

² Professor, Department of Clinical Microbiology, Christian Medical College and Hospital, Vellore, Tamil Nadu, India.

³ Instructor, Department of Clinical Microbiology, Christian Medical College and Hospital, Vellore, Tamil Nadu India.

⁴ Professor and Head, Department of Clinical Microbiology, Christian Medical College and Hospital, Vellore, Tamil Nadu, India.

⁵ Professor, Department of International Health, and Department of Paediatrics, Johns Hopkins Medical Institutions, Baltimore, MD, USA.

⁶ Indian Council of Medical Research Emeritus Scientist, Department of Clinical Virology, Christian Medical College and Hospital, Vellore, Tamil Nadu, India.

India. Clinical samples and/or bacterial strains were sent from these centres to Vellore for confirmation of *S. pneumoniae*, including serotype and antibiotic susceptibility. All strains were preserved by lyophilization or freezing at -70°C .

An essential component of this prospective surveillance study was the ability to preserve pneumococcal isolates reliably for comparatively long periods of time. *S. pneumoniae*, being a fragile organism, does not survive well in broth cultures in hot climates and can survive on culture plates for only two or three days. While freezing at -70°C and lyophilization are recognized to be effective techniques, the high cost and difficulty in acquiring and maintaining equipment and test materials prevent their use in low-income regions of the world. We evaluated alternative low-cost techniques using locally available products against the standard techniques of -70°C freezing and lyophilization. In order to identify suitable methods for the preservation of bacterial isolates for tests in developing countries, this study evaluated a variety of media and storage temperatures for the preservation of *S. pneumoniae* in a tropical setting.

Materials and methods

Bacterial strains

Cultures of *S. pneumoniae*, isolated from clinical samples in the Department of Clinical Microbiology, Christian Medical College and Hospital, Vellore, Tamil Nadu, India, were used for the study. We included capsular types 1, 5, 7, 19, and 23 — representing the types commonly encountered in both India and the USA — which were identified and serotyped by methods standardized in our laboratory (5). The isolates were subcultured on trypticase soy blood agar and then subjected to different methods of preservation as described below.

Freezing in glycerol–chocolate broth

Samples (0.5 ml) from an overnight glycerol–chocolate broth culture, supplemented with sterile glycerol (final concentration, approximately 8.7% v/v), were placed in freeze-resistant storage vials (Provia, Laxbro, Pune) and stored at -20°C or -70°C .

Freezing in skimmed milk

Samples (0.5 ml) of suspensions, made from harvesting overnight cultures on sheep blood agar slopes with 1.0 ml sterile skimmed milk, were dispensed in storage vials and stored at -20°C or -70°C .

Freezing in rabbit blood

Samples (0.5 ml) of culture suspension, obtained by harvesting overnight growth from sheep blood agar slopes with 1.0 ml sterile defibrinated rabbit blood, were placed in storage vials and stored at -20°C or -70°C .

Freezing in sheep blood

Samples (0.5 ml) of culture suspension obtained as described for rabbit blood, but harvested with 1.0 ml

sterile defibrinated sheep blood, were placed in storage vials and stored at -20°C or -70°C .

Lyophilization

This was performed using Edwards freeze-drying equipment (Edwards High Vacuum International, Essex, England) according to the manufacturer's instructions. Cultures grown on blood agar slopes overnight were washed with 1.0 ml sterile skimmed milk. A few drops of this suspension were transferred into vials and lyophilized. Vials were flamed-sealed under vacuum and stored at room temperature or at 4°C .

Sand desiccation

This was carried out by the method described earlier (6–8). Briefly, overnight cultures of organisms grown on blood agar slopes were washed with 0.5 ml of sterile defibrinated sheep blood and mixed well with fine, filtered, sterile river-bed sand (Palar River, N. Arcot District, TN, India), dispensed in cotton-plugged Durham's tubes (M/S Sush-Lab Corporation, Mumbai, India). The mixed sand particles were layered along the walls of the tubes to form a thin layer. Care was taken not to form clumps of sand particles in the tubes. Multiple tubes of the five serotypes were arranged in small bundles and placed in sterile, wide-mouthed bottles containing silica gel. The bottles were closed airtight using a vacuum, and were stored at room temperature or 4°C .

Quantification of specimen viability

Initial quantification. A representative inoculum from each of the five serotypes preserved by various methods was quantified on trypticase soy agar plates; 0.01 ml bacterial suspension was diluted in 9.99 ml of sterile physiological saline (0.85% NaCl w/v, pH 7.0), and 1 μl of the diluted suspension was spread on trypticase soy agar plates and incubated at 37°C in a candle extinction jar for 16–18 hours. The number of colonies on the plates multiplied by 10^6 gave the initial colony count/ml.

Quantification at different intervals. The viability of preserved strains was checked on day 0 and after 1 week, 4 weeks, 4 months and 16 months by subculturing them on trypticase soy agar plates. Two methods of subculturing were performed. In one, 0.01 ml of the thawed suspension was diluted in 9.99 ml sterile physiological saline (0.85% NaCl w/v, pH 7.0) and 1 μl of this was spread on Mueller–Hinton blood agar plates. In the second, 0.01 ml of the thawed suspension was diluted in 0.99 ml of sterile physiological saline (0.85% NaCl w/v, pH 7.0), and 1 μl of this diluted suspension was spread on Mueller–Hinton blood agar plates. Colonies were counted on duplicate plates after overnight incubation at 37°C in CO_2 . Lyophilized samples were reconstituted in 0.5 ml glycerol–chocolate broth and then diluted as above. For sand-desiccated tubes, a few grains of sand particles were suspended in 1.0 ml glycerol–chocolate broth and subcultured on Mueller–

Hinton blood agar plates. The presence or absence of growth on the plate was recorded, but quantification by colony counts was not possible for the sand-preserved specimens. Pneumococcal capsular types were confirmed by a coagglutination method (4). After the initial set of subcultures was tested for 16 months, the sand-desiccated tubes were further tested for viability at 6-monthly intervals over a period of 5 years and 8 months. The last subculture was carried out in November 1999.

Temperature monitoring

Room temperature in the laboratory and the official Vellore Meteorological Office minimum and maximum temperatures for each day were recorded. The internal temperature of the freezers and refrigerators were recorded every morning.

Results

The colony counts of all strains stored in glycerol–chocolate broth and skimmed milk at 4 °C had declined by several thousand-fold, by the end of the first week (glycerol–chocolate broth) or fourth week (skimmed milk) (Fig. 1). Strains preserved in glycerol–chocolate broth, skimmed milk, rabbit blood, and sheep blood at 4 °C became nonviable by the fourth month. In contrast, all strains stored at –70 °C were found to be viable at 16 months. The decline in the colony counts over the 16 months varied from 5-fold (sheep blood) to 92-fold (glycerol–chocolate broth).

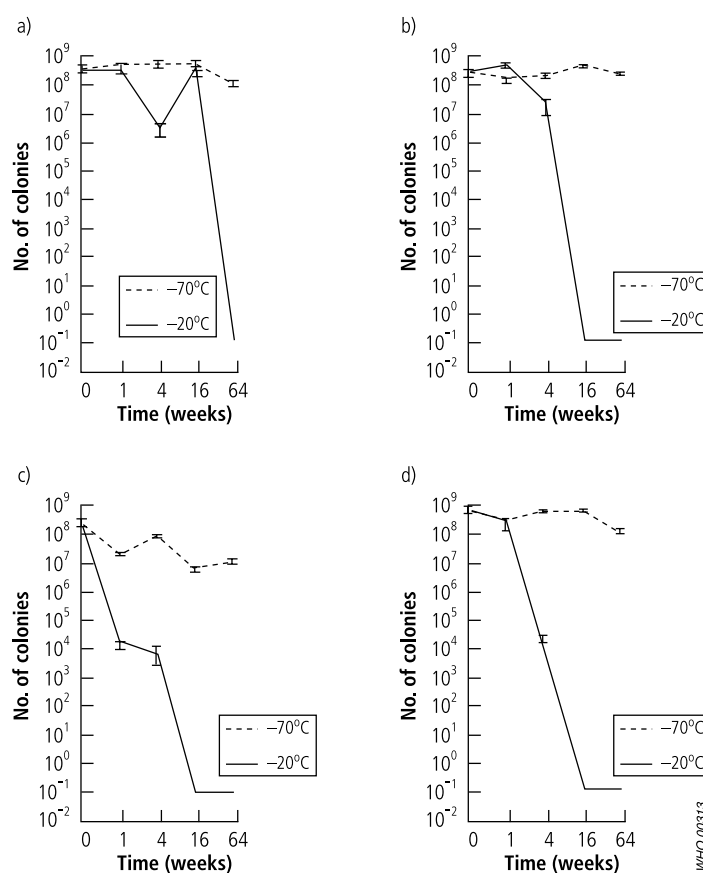
Fig. 2 compares the effects of storing lyophilized cultures at room temperature and at 4 °C. At room temperature, the colony counts declined by 1000-fold by the fourth week to become nonviable by the fourth month. At 4 °C, the cultures were still viable even at 16 months, though the colony count had declined 10-fold. Specimens preserved by sand desiccation and stored at room temperature lost their viability by the fourth week, while those stored at 4 °C were found to be viable for up to 5 years and 8 months.

Discussion

Our evaluation of a variety of methods for preserving *S. pneumoniae* confirms the need for lyophilization or storage at –70 °C in order to keep this fragile organism viable in hot and humid climates. Preservation at –20 °C was found to be inadequate, as none of the strains was viable after 16 months. Interestingly, sand desiccation and storage at 4 °C proved to be a very effective method, as shown by viability of all strains at 16 months, thus validating desiccation as an effective method for preserving *S. pneumoniae*.

Long-term preservation of *S. pneumoniae* in laboratories in low-income developing countries poses significant problems in terms of cost and maintenance of the equipment. High environmental temperatures during summer months and the fragile

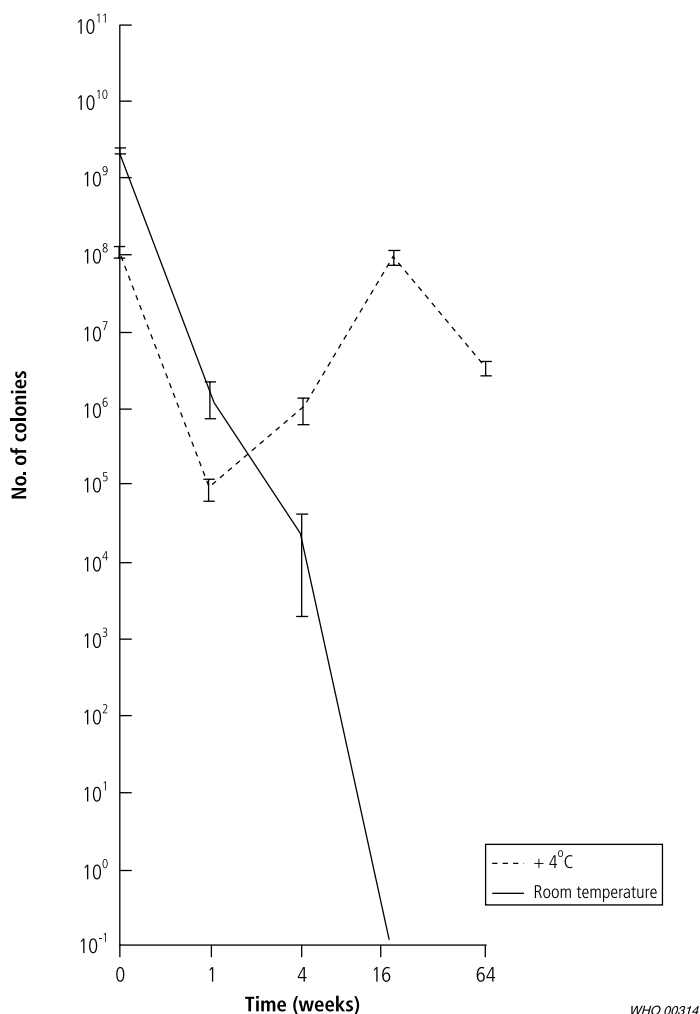
Fig. 1. Number of *S. pneumoniae* colonies versus time after preservation in a) rabbit blood, b) sheep blood, c) glycerol–chocolate broth, d) skimmed milk



nature of this organism make it difficult to preserve it for more than a few days. Escalating costs, inadequate equipment maintenance, and irregular power supply are important factors which make –70 °C freezing and lyophilization facilities unavailable to many laboratories in developing countries. Therefore, development of appropriate techniques which can easily be standardized will be advantageous for the long-term preservation of *S. pneumoniae* in such countries.

WHO has listed several media in which *S. pneumoniae* can be suspended for preservation by freezing: these include skimmed milk, and defibrinated horse or rabbit blood, but not sheep blood (9). In the present study, preservation of *S. pneumoniae* under different culture conditions at –20 °C showed that none of the strains was viable up to 16 months. The WHO manual states that storage at –20 °C will result in some loss of viability, and our results confirm this. In contrast, storage at –70 °C was found to be an ideal method for the preservation of these organisms in rabbit or sheep blood as well as in skimmed milk, with all three being equally effective at –70 °C. Loss of viability at –70 °C, as indicated by a fall in colony counts, was minimal in different media although the decline was more pronounced in glycerol–chocolate broth (Fig. 1). These results suggest that temperature is a more critical factor than the medium for the preservation of *S. pneumoniae*.

Fig. 2. Number of *S. pneumoniae* colonies versus time after preservation of lyophilized cultures at room temperature and 4°C



Lyophilization with storage at 4 °C can preserve streptococci for more than 20 years, with over 95 % viability (10). Some species of streptococci have been shown to have nearly equivalent survival rates when lyophilized cultures were stored at room temperature instead of 4 °C. Recently, Wasas et al. (11) described the usefulness of Dorset agar medium for the preservation of *S. pneumoniae*. When preserved on this medium at room temperature (21 °C), colonies of *S. pneumoniae* were viable for up to 44 days, compared with only 30 days when the medium was supplemented Columbia agar base. They concluded that Dorset agar medium, which is inexpensive, is ideal for the

transportation of *S. pneumoniae* between laboratories, but it is not known if such cultures will remain viable when the room temperature goes up to 40 °C. In the present study, storage of lyophilized or sand desiccated cultures at room temperature adversely affected the viability of cultures at 4 months and longer. This is of importance in countries like India, where the environmental temperature in the summer months can reach 40–44 °C. Storage at 4 °C, however, kept the strains viable for up to 16 months with very little loss of viability. Our data suggest that in hot and humid climates, lyophilized or sand desiccated cultures of *S. pneumoniae* need to be stored at refrigerator temperatures for their long-term preservation.

A variety of factors are responsible for the difficulty of preserving fastidious and fragile organisms in developing countries. Therefore methods have to be developed which can utilize locally available technology and facilities. We have earlier shown that group A *S. pyogenes* can be successfully preserved for as many as 22 years by the sand desiccation technique (7). The present study shows that *S. pneumoniae* can be preserved by the latter method without any loss of viability for at least 16 months. We have recently observed that sand desiccation and storage at 4 °C can preserve *S. pneumoniae* for 42 months (8). Materials required for this method are locally available, while the technique itself is of low cost and is easy to perform. Recovery of organisms is possible from a few sand particles and the original tubes can be used again after storage. This technique does not require expensive technology or additional expertise and is low in maintenance requirements. In many tropical countries including India, the environmental/room temperatures may reach as high as 44 °C. We do not know if organisms will remain viable on Dorset agar (11) at such high temperatures. On the other hand, we have shown that *S. pneumoniae* does not remain viable after sand desiccation and storage at high room temperature, but this technique does preserve these organisms at 4 °C. The last subculture, performed in November 1999, has shown that *S. pneumoniae* can remain viable by this method up to 68 months when stored at 4 °C. We conclude that sand desiccation, which is within the technical and financial reach of most diagnostic laboratories, can be recommended as an alternative and cost-effective method for the long-term preservation of *S. pneumoniae* in developing countries. ■

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Résumé

Comparaison de différents milieux de culture et températures de stockage pour la conservation prolongée de *Streptococcus pneumoniae* en milieu tropical

Objectif La conservation de *Streptococcus pneumoniae* par les techniques classiques de congélation en vue de tests ultérieurs tels que sérotypage et antibiogramme est difficile voire impossible dans de nombreux pays en

développement en raison du coût élevé de l'équipement, de l'insuffisance de la maintenance et de l'irrégularité de l'alimentation électrique. Nous avons évalué des méthodes de remplacement peu coûteuses en compa-

rant différents milieux de culture et températures de stockage.

Méthodes Des isollements cliniques appartenant à cinq types capsulaires (1, 5, 7, 19 et 23) de *S. pneumoniae* ont été conservés dans du sang de lapin, du sang de mouton, du lait écrémé ou du bouillon chocolat glycérolé et stockés à -20°C ou -70°C . Des cultures ont aussi été conservées par lyophilisation et par dessiccation sur sable et stockées à température ambiante ou à 4°C . La viabilité des cultures conservées a été déterminée par numération des colonies le jour 0 et au bout de 1 semaine, 4 semaines, 4 mois et 16 mois. La viabilité des cultures desséchées sur sable et stockées à 4°C a également été déterminée tous les 6 mois jusqu'à 68 mois.

Résultats Quels que soient les milieux utilisés, les cultures stockées à -20°C perdaient leur viabilité au bout de 4 mois tandis que celles stockées à -70°C étaient encore viables au bout de 16 mois. Les cultures conservées par lyophilisation et par dessiccation sur sable perdaient leur viabilité au bout de 4 mois lorsqu'elles étaient stockées à température ambiante ($30-42^{\circ}\text{C}$) mais restaient viables jusqu'à 68 mois après stockage à 4°C .

Conclusion Nos résultats confirment que la congélation à -70°C et la lyophilisation suivie d'un stockage à 4°C sont les méthodes idéales de conservation de *S. pneumoniae*. Dans les laboratoires non équipés pour la lyophilisation, la dessiccation sur sable suivie d'un stockage à 4°C constitue une alternative peu coûteuse pour la conservation prolongée de ce micro-organisme.

Resumen

Comparación de diferentes medios de cultivo y temperaturas de almacenamiento para la conservación prolongada de *Streptococcus pneumoniae* en zonas tropicales

Objetivo La conservación de *Streptococcus pneumoniae* mediante los métodos estándar de congelación con miras a posteriores análisis — como las pruebas de serotipificación y de sensibilidad a antibióticos — resulta ardua o imposible en muchos países en desarrollo debido al alto costo del equipo, a las dificultades para mantenerlo y a la irregularidad del suministro eléctrico. Hemos evaluado diversos métodos de bajo costo alternativos, comparando diferentes medios de cultivo y temperaturas de almacenamiento.

Métodos Los aislamientos clínicos de cinco tipos capsulares (1, 5, 7, 19 y 23) de *S. pneumoniae* fueron conservados en sangre de conejo, sangre de carnero, leche desnatada o caldo de glicerol-chocolate, y almacenados a -20°C o -70°C . Los cultivos se conservaron asimismo mediante liofilización o por desecación con arena, seguida de su almacenamiento a temperatura ambiente y a 4°C . La viabilidad de los cultivos se determinó mediante recuentos sucesivos del número de colonias, el día 0 y al cabo de 1 semana, 4 semanas, 4 meses y 16 meses. La viabilidad de los

cultivos conservados mediante desecación con arena y su posterior almacenamiento a 4°C se determinó también cada 6 meses, por espacio de hasta 68 meses.

Resultados Independientemente de los medios utilizados, los cultivos mantenidos a -20°C dejaron de ser viables al cuarto mes, mientras que los mantenidos a -70°C eran todavía viables al cabo de 16 meses. Los cultivos conservados mediante liofilización o desecación con arena dejaron de ser viables al cuarto mes cuando se mantuvieron a la temperatura ambiente local ($30-42^{\circ}\text{C}$), pero almacenados a 4°C su viabilidad no decayó por espacio de hasta 68 meses.

Conclusiones Nuestros resultados confirman que la congelación a -70°C y la liofilización y el posterior almacenamiento a 4°C son los métodos ideales para la conservación de *S. pneumoniae*. En los laboratorios donde no es posible liofilizar las muestras, la desecación con arena y el almacenamiento a 4°C constituye un método alternativo de bajo costo para la conservación a largo plazo de *S. pneumoniae*.

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