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Quantitative Validation of Media for Transportation and Storage of *Streptococcus pneumoniae*

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The need to design effective *Streptococcus pneumoniae* vaccines and to monitor resistance means that it is essential to have efficient methods to determine carriage rates. Two liquid media, consisting of skim milk, glycerol, glucose, and tryptone soya broth (STGG) or skim milk, glycerol, and glucose (SGG) alone, were evaluated for their ability to maintain pneumococcal viability. Optimal recovery of *S. pneumoniae* was achieved when swabs were transferred to STGG medium prior to plating onto blood agar-gentamicin selective plates (22%) compared to 7% when plated out directly ($P < 0.0001$ by Fisher's exact test). Both STGG and SGG media are appropriate for the long-term storage of pneumococci and primary swab samples at −70°C, with no decrease in viable count observed following repeated freeze-thaw cycles. Samples could be stored refrigerated for up to 3 days in either STGG or SGG medium with no significant loss of viability. Viability decreased progressively in storage at 20 to 30°C, with greater losses of viability occurring at the higher temperatures. There were no significant differences in viability between isolates in the two media. STGG preserved pneumococci significantly better (about twofold) than SGG medium at 21°C ($P < 0.0001$) and 30°C ($P < 0.0001$). Samples can be stored for 4 and 2.5 days at 6 to 8°C, 28 and 17 h at 21°C, and 15 and 7 h at 30°C in STGG and SGG media, respectively. For field studies undertaken in resource-limited environments, SGG medium can be prepared by using locally available materials. The quantitative data reported in this study will enable researchers to plan appropriate transport and storage protocols.

An estimated three to five million deaths occur annually in children under 5 years of age due to acute respiratory infections, for which *Streptococcus pneumoniae* is the most important pathogen (9, 17). *S. pneumoniae* normally colonizes the human nasopharynx, nose, and throat asymptomatically. The incidence of pneumococcal disease and the occurrence of antibiotic-resistant isolates have been positively correlated to the levels of carriage (3, 7, 15, 19). Carriage can be influenced by age (highest in infants and decreasing with age), immune status, seasonal variation, socioeconomic factors, and other demographic features (8).

Most carriage data on pneumococcal serotype distribution, age-specific disease rates, and prevailing antibiotic sensitivities are usually obtained from developed countries, but information on the distribution of pneumococcal serotypes and antibiotic sensitivities in developing countries is essential for the design of vaccines and for optimal treatment where the burden of disease is greatest. The risk of serotype switching and increasing resistance means that this information must be constantly updated (2, 11).

For meaningful surveillance studies to be undertaken regarding pneumococcal carriage and disease, it is essential to ensure that primary swab samples are processed efficiently with optimal recovery of pneumococci and that the transport and storage medium maintains the viability of the swab samples without introducing bias that would skew the data.

One medium that may be appropriate for these studies, STGG (consisting of skim milk, tryptone soya broth, glycerol, and glucose), was used in some field studies of pneumococcal carriage (12). This medium was evaluated for the optimal recovery and transport of *S. pneumoniae* isolates by qualitative and semiquantitative methods (18). This study showed that the recovery of pneumococci from primary swabs transferred to STGG medium was equivalent to the recovery seen with direct plating. Transporting and storing primary swabs in chilled liquid media allows samples to be processed in batches back in the laboratory, allows multiple samples to be processed from each swab, and permits long-term storage of primary swabs and isolated pneumococci. However, this study did not determine the viability of different pneumococcal isolates or serotypes in STGG medium that may introduce bias in carriage data. In addition, transportation on ice may not always be feasible, and no evaluation of the viability of pneumococci at higher ambient temperatures in this medium was performed. Furthermore, the effect of repeated freeze-thaw cycles was not investigated. Another study of several culture media and storage temperatures for long-term preservation of *S. pneumoniae* undertaken in the tropics showed that archiving pneumococci at −70°C in skim milk alone was sufficient to maintain pneumococcal viability for at least 64 weeks (21).

The purpose of this study was to compare the recovery of pneumococci by direct plating of swabs and after resuspension of the swab in STGG in order to evaluate the viability of different pneumococcal isolates in STGG at various tempera-

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tures and to compare their viability in medium lacking tryptone (i.e., SGG). Archiving bacteria in an ordinary freezer at around −20°C can produce variable results. In general, this temperature is not recommended for preserving bacteria because they can be damaged by the eutectic effect, as two or more miscible substances liquefy at the lowest temperature of all such mixtures (4, 13). Therefore, we did not assess SGG and STGG media at this temperature. Tryptone soya broth is an expensive reagent, costing $30/500 g in the United Kingdom, that may not be available in regions where field studies are performed. This study demonstrates that there were no significant differences in viability of *S. pneumoniae* between different isolates in either STGG or SGG medium. In addition, a constant rate of decline in viable count was observed at each of the temperatures above −70°C tested. The rate constants in STGG were half those in SGG. From these data, the viability of cells stored at various ambient temperatures in either STGG or SGG could be calculated.

MATERIALS AND METHODS

Subjects. Throat swabs were obtained from children under 7 years of age who were attending outpatient clinics at Tanganyika Plantation Company in Moshi, Northern Tanzania.

Ethics and informed consent. The study was approved by the local ethics committee and the National Institute for Medical Research in Tanzania. Written informed consent was obtained from parents or legal guardians.

Preparation of STGG and SGG media. STGG medium consisted of 3% (wt/vol) tryptone soya broth (CM 129; Oxoid), 0.5% (wt/vol) glucose (Sigma), 2% (wt/vol) skim milk powder (CM L310; Oxoid), and 10% (vol/vol) glycerol in distilled water (18). SGG has the same composition as STGG but without tryptone soya broth. One-milliliter aliquots were dispensed into 1.8-ml cryotubes (Nunc) for storage of *S. pneumoniae* at −70°C, or 4-ml aliquots were dispensed into 7-ml glass sample containers (Bijoux) for viability counts. We were careful to mix each medium at regular intervals. The media were sterilized by either autoclaving at 10 lb/in^2 for 10 min or by tyndallization (repeated steaming at 100°C followed by overnight culture at 37°C). The sterilized media were refrigerated (6 to 8°C) until further required (5). Prior to use, the pellet in the bottom of the tube was resuspended by vortexing for 10 to 15 s. Sterility testing was performed after each instance of medium preparation.

Sample collection and processing. Oropharyngeal swabbing was used to sample for carriage of pneumococci (7), as discussions with the village elders and with the ethics committee indicated that this method would be less distressing than nasopharyngeal swabbing for children under 7 years of age; oropharyngeal swabbing also ensured better compliance. Throat swabs (Dacron cotton wool buds; Medical Wire & Equipment Co., Ltd., Corsham, United Kingdom) were taken and either plated immediately onto selective Columbia agar with 5% (vol/vol) defibrinated sheep blood and 5 μg of gentamicin per ml (BAG) by spreading the swab over an area of about 2 cm^2 and then streaking out with a sterile loop or placed in 1.0 ml of sterile STGG in a 7.0-ml plastic Bijoux (screw-cap specimen container). The wooden handle of the swab was cut off with sterilized scissors to fit inside the Bijoux, and the screw-cap was tightened. The Bijoux was vortexed for 10 to 20 s to transfer the organisms from the swab into the STGG medium. Aseptically, the swab was squeezed against the side of the Bijoux in order to leave as much of the medium in the bottle as possible before the swab was removed. After 2 to 3 h at ambient temperature, 10 microliters (1%) of the resuspended swab sample was inoculated onto BAG plates. Plates were incubated aerobically at 37°C for 48 h. The remaining unprocessed sample was transferred aseptically to 1.8-ml sterile cryotubes and archived at −70°C. If samples were negative or if only a few organisms were present, 100 μl (10%) was plated onto BAG plates and cultured as just described.

Pneumococcal identification. Presumptive α-hemolytic colonies with morphologies consistent with pneumococci were confirmed by Optochin sensitivity. Isolated pneumococci were purity plated and archived in triplicate in 1.0 ml of STGG medium and stored at −70°C.

Viable count. Archived pneumococcal isolates were subcultured from frozen STGG or SGG medium onto five blood agar plates and incubated overnight at 37°C in 5% CO_2. Cells were harvested from four or five plates, depending on the density of growth, with a sterile cotton wool bud and suspended in 4.0 ml of either STGG or SGG medium. As a baseline control, viable counts were performed according to the method of Miles and Misra (16) at this time. One-milliliter aliquots were then placed at either 30°C, room temperature (21°C), 4 to 8°C (refrigerated), or −70°C, and viable counts were performed at 24-h intervals to obtain survival curves as follows. Samples were mixed by brief vortexing, and log dilutions to 10^{-6} in sterile distilled water were set up. Twenty microliters of each dilution was spotted onto a blood agar plate and dried on a flat surface. The plates were incubated overnight at 37°C in 5% CO_2, and then the number of colonies was counted. Each determination was made in triplicate and expressed as the mean number of CFU per milliliter ± standard error of the mean.

Statistical methods. Statistical analysis was performed by using the two-sided Fisher's exact test for calculating the significance level between the two methods of dealing with the primary oropharyngeal swabs. For calculating the significance level between the numbers of viable pneumococcal cells (expressed as CFU per milliliter) at different temperatures and analyzing differences between isolates of the same serotype, a two-tailed unpaired *t* test with Welch's correction was performed by using version 3.00 for Windows (http://www.graphpad.com; GraphPad Software, San Diego, Calif.). The viable counts were fitted by least-squares error analysis to a monophasic exponential decay curve by using GraphPad Software. As the viable count would always eventually reach zero, the plateau was fixed at this point. Data were fitted to the following equation:

$$V_t = v_0 \cdot e^{-kt}$$

where V is the viable count (CFU/ml), v_0 is the viable count at time zero, t is time, e is the Napierian constant, and k is the exponential decay constant.

The correlation coefficient (r^2) was calculated as an estimate of goodness of fit by using GraphPad Prism software as follows:

$$r^2 = \frac{\text{(sum of squares of the distance from the best-fit curve)}}{\text{(sum of squares of distance from the mean } y \text{ value)}}$$

The half-life was calculated from the following equation:

$$t_{1/2} = \frac{\ln (0.5)}{k}$$

To determine whether the differences in viability of the isolates were significant, their $t_{1/2}$ values were analyzed by a one-way analysis of variance that was performed by using GraphPad software. Differences in $t_{1/2}$ between STGG and SGG media were analyzed by a two-tailed unpaired *t* test with Welch's correction (GraphPad Software).

RESULTS

Recovery of pneumococci from primary oropharyngeal swabs. Oropharyngeal samples from healthy children under 7 years of age were obtained from children undergoing routine health checks at the Tanganyika Plantation Company Hospital, Moshi, Northern Tanzania. Of 82 oropharyngeal swabs transferred to STGG prior to plating, 18 were pneumococcus positive (22%). Of 211 samples plated directly onto BAG plates, 15 were positive for pneumococci (7.1%). The difference was highly significant ($P = 0.0007$ by Fisher's exact test).

Viability of different pneumococcal isolates stored in STGG medium at various temperatures. The viability of two different isolates of each of the pneumococcal serotypes, i.e., 6, 18, 19, and 23, were evaluated quantitatively in STGG medium. At −70°C, all isolates maintained their viability after four freeze-thaw cycles (Fig. 1A). At higher storage temperatures, only isolate 107 (serotype 18) showed significant differences ($P < 0.05$) from isolate 9 (serotype 18) at some time points (Fig. 1B and C). No significant differences in the $t_{1/2}$ value (index of the viability) of each isolate were observed between isolates of the same and different serotypes for each storage temperature tested. As the storage temperature was increased from 4 to 8°C up to 30°C, a decrease in viability was observed for all isolates (Fig. 1B through D).

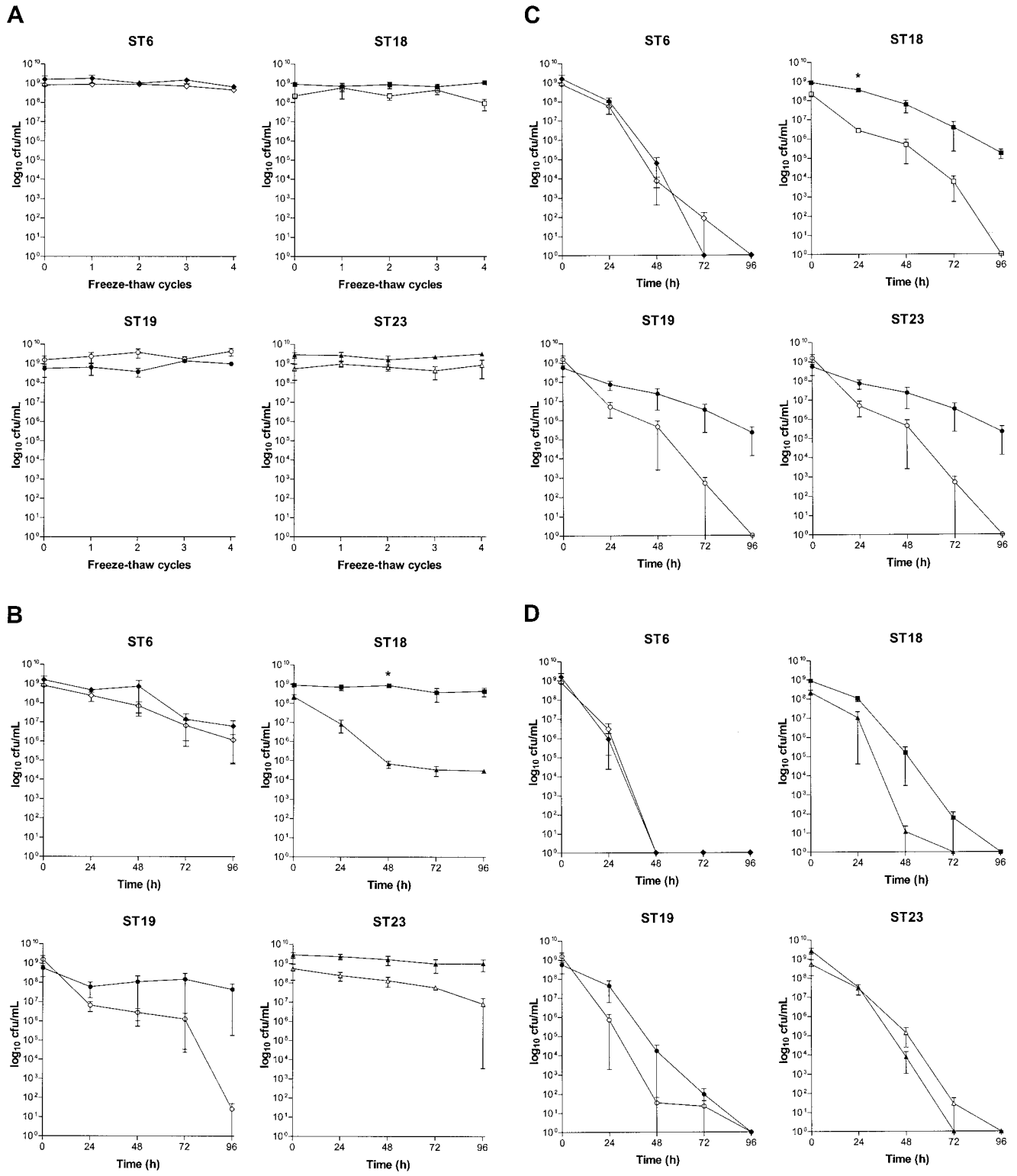

FIG. 1. The viability of two different isolates of each serotype (ST) stored in STGG medium at different temperatures. Pneumococcal cells from overnight cultures were transferred to 4.0 ml of STGG medium, and 1-ml aliquots were stored frozen at −70°C (A) and refrigerated at 6 to 8°C (B), 21°C (C), and 30°C (D). Frozen samples were thawed at each sampling point and frozen immediately afterward. Two different isolates (Royal Free numbering) were tested for each capsular ST: ST 6 no. 10 (♦) and 156 (◇), ST 18 no. 9 (■) and 107 (□), ST 19 no. 5 (●) and 25 (○), and ST 23 no. 6 (▲) and 13 (△). Data are shown as the means of results from three independent experiments, and error bars indicate standard errors of the means. Each experiment was performed in triplicate.

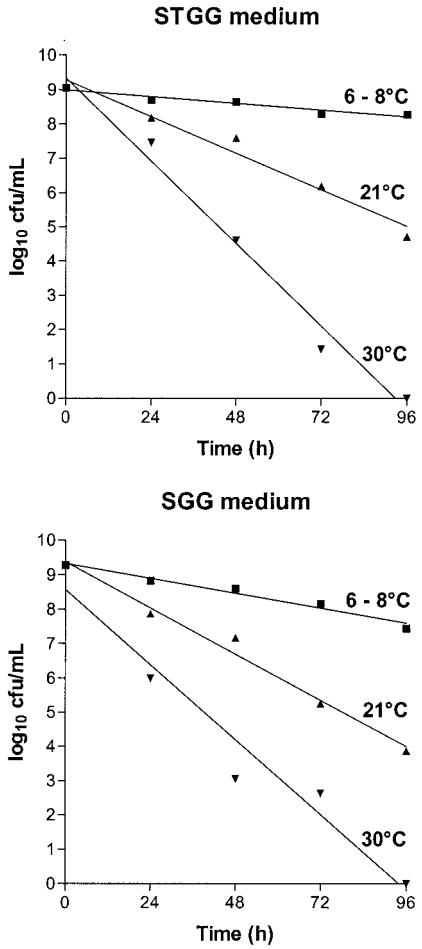

FIG. 2. Pneumococcal cell viability of serotypes 6, 18, 19, and 23 stored in either STGG medium or SGG medium at various temperatures. Pneumococcal cells from overnight cultures were transferred to 4.0 ml of STGG medium (upper panel) or 4.0 ml of SGG medium (lower panel), and 1-ml aliquots were stored refrigerated at 6 to 8°C, at 21°C, or at 30°C. Although $t_{1/2}$ values were determined from nontransformed data, the $\log_{10}$ of viable cell counts is plotted against storage time for each temperature for clarity, as indicated. Each data point is the mean value for all of the isolates studied ($n = 12$).

The viability of pneumococcal cells stored in either STGG or SGG medium. As no significant differences were observed between the study isolates that represented serotypes 6, 18, 19, and 23, they were grouped together to compare the cell viability during storage at various temperatures in either STGG or SGG medium.

At storage temperatures above −70°C, the viable count decreased by a constant rate that was described by a monophasic exponential decay equation (Fig. 2). Cells stored in SGG medium had a lower viability than did cells stored in STGG medium. The $t_{1/2}$ value at 4 to 8°C was 1.6-fold lower in SGG medium, 1.7-fold lower at 21°C ($P < 0.0001$), and 2.1-fold lower at 30°C ($P < 0.0001$) (Table 1).

As a guide for storing pneumococcal cells during transit in either STGG or SGG medium at temperatures above −70°C, the time taken to reduce the viable cells by 1 log was calculated from the $t_{1/2}$ values in Table 1, and these reduction times are recorded in Table 2.

TABLE 1. The viability of pneumococci stored in either STGG or SGG medium at different temperatures[a]

Storage temp (°C)	Result with: STGG medium $t_{1/2} \pm$ SEM ($n = 12$) (h)	STGG medium r^2	SGG medium $t_{1/2} \pm$ SEM ($n = 12$) (h)	SGG medium r^2	Significance level[b]
6–8	30.91 ± 5.14	0.976	24.91 ± 2.27	0.999	NS
21	8.43 ± 0.28	1.000	5.14 ± 0.15	1.000	$P < 0.0001$
30	4.53 ± 0.02	1.000	2.20 ± 0.0001	1.000	$P < 0.0001$

[a] The mean viable counts of all pneumococcal isolates were fitted by nonlinear-regression analysis to the monophasic exponential decay equation, $V_t = \text{CFU/ml } (t_0) \times e^{-kt}$, and the half-life is ln (0.5)/k, where V represents viable cell count (CFU/ml) and t represents time (hours). r^2 is the correlation coefficient. Each isolate was tested in three independent experiments, each of which was performed in triplicate.

[b] Significance between STGG and SGG media calculated by a two-tailed unpaired t test with Welch's correction (NS, not significant).

DISCUSSION

In pneumococcal studies of carriage, different swabbing sites and isolation methods may cause variations in sensitivities. Early studies to identify optimal sites for swabbing indicated that there was little difference between oro- and nasopharyngeal swabbing (10, 14, 22). More recent studies indicated that the anterior nares may be a better site for swabbing than the oropharynx (1). In this study, swabs were placed in Amies transport medium and either plated directly or transported to the laboratory with a transit time of 6 to 7 h at ambient temperature. The plate medium was trypticase soy agar containing 5% BAG. The overall carriage rates observed upon immediate inoculation at the site of sampling were similar to those found when inoculation was carried out after transport to the laboratory. In 1997, Rapola et al. compared nasopharyngeal aspirates and nostril, nasopharyngeal, and oropharyngeal swabs and observed carriage rates of 33, 32, 30, and 20%, respectively (20). In this study, all specimens were placed in Stuart's medium and stored overnight at 4°C before being plated onto BAG plates. It is not clear why the earlier studies of sampling methods appear to show fewer differences between

TABLE 2. Recommended maximum storage periods at various temperatures of pneumococcal samples in either STGG or SGG medium[a]

Storage temp (°C)	Medium	Storage period (h)[b]
6–8	STGG	102.6
6–8	SGG	60.6
21	STGG	28.1
21	SGG	16.9
30	STGG	15.1
30	SGG	7.3

[a] The means ± standard errors of the means of the viable cell counts of all the pneumococcal isolates studied were fitted by nonlinear-regression analysis to a monophasic decay curve that enabled a $t_{1/2}$ value to be calculated. From each curve, the time taken to decrease the viable cell count by 1 log was determined.

[b] This figure is based on the time taken to decrease the viable cell count by 1 log.

oropharyngeal and nasopharyngeal sampling than the more recent studies. Variations in methodology prior to plating out may have been responsible for this discrepancy, as all of the studies isolated pneumococci on BAG plates.

Carriage studies are frequently undertaken for situations in which immediate processing of the samples is not possible. Few investigations, which are essential for planning carriage studies, have been undertaken to characterize the performance of transport medium, whereas several studies have investigated storage medium used for archiving samples (5, 21, 23). A transport medium containing STGG, which had previously been used only to archive samples at −20 and −70°C (6, 12), was recently evaluated for the transportation of pneumococci at 4°C (18). This study confirmed that samples could be frozen in STGG and determined that STGG was an appropriate medium for the immediate transfer of nasopharyngeal swab flora and transportation at 4°C. Transfer into STGG yielded results similar to those obtained when plating out directly onto BAG (18). Our study differs from this finding in that our results show superior performance for delayed plating. Significantly higher recoveries were observed when swabs were transferred to STGG medium prior to plating onto BAG than when swabs were plated directly onto identical blood plates. This would indicate that (i) there is either more efficient transfer of pneumococci from a swab to liquid medium than is possible for direct plating from a swab or (ii) because pneumococci are prone to rapid desiccation, they remain more viable if immediately transferred to STGG. Our study was performed in a hot (around 30°C) and dry rural environment, so it is likely that plating of pneumococci from a dry swab leads to some loss in viability. We also transferred only 1% of the sample, allowing for repeats or transfer of 10% if the plates were negative or contaminated. The higher yield and the efficiency gains of this method suggest that it should be adopted for pneumococcal carriage studies.

In this study, the performance characteristics are defined for both STGG and SGG media at 4, 21, and 30°C. Pneumococcal viability was also tested over multiple freeze-thaw cycles that may arise if a freezer is located in a region where electrical power failures are frequent. Both STGG and SGG media maintained pneumococcal viability over several cycles of freeze-thawing, indicating that both media are suitable for long-term storage. This confirms previous findings that tryptone soya broth's use in archiving medium is not essential for maintaining the viability of pneumococcal cells stored at −70°C (21).

The rates of decline in viable counts were established, and although some variations were observed between isolates, no significant differences were observed between different isolates representing serotypes 6, 18, 19, and 23 in STGG or SGG medium at each temperature tested. However, the viability of pneumococci was approximately twofold better in STGG medium than in SGG medium, attaining significance at 21 and 30°C ($P < 0.0001$). From the measured rates of decline in viable counts, the period of storage at 4, 21, and 30°C can be calculated, and this permits strategies for collection and transport of specimens to be planned with confidence.

In conclusion, optimal recovery of pneumococci occurs when samples are transferred to liquid media rather than plated out directly; both STGG and SGG media are appropriate for use in studying pneumococcal carriage; and the quantitative evaluation undertaken in this study will allow field studies to be designed depending on the resources available.

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