Optimisation of storage conditions for maintaining culturability of penicillin-susceptible and penicillin-resistant isolates of *Streptococcus pneumoniae* in transport medium

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ABSTRACT

Methods employed by the World Health Organization (WHO) are used during this study to determine the optimum storage conditions for maintaining the culturability of *Streptococcus pneumoniae* in skimmed milk, tryptone, glucose and glycerin (STGG) transport medium. A comparison of *S. pneumoniae* strains sensitive and resistant to penicillin showed no significant difference in their survival ability in STGG medium. Furthermore, it is confirmed that storage at –70°C remains most effective for maintaining viability by culture of *S. pneumoniae*. Storage at –20°C would only be acceptable in the short-term, while storage at +4°C is not recommended. Of note, this study has shown STGG medium at room temperature to be an efficient growth medium for pneumococci in the short-term. This work will help to establish robust sampling protocols when performing community studies to ensure culturability of comparison between community and laboratory pneumococci survival.

KEY WORDS: Ciprofloxacin.
Penicillin.
Streptococcus pneumoniae.
STGG medium.
Survival.

Introduction

Streptococcus pneumoniae is a major pathogen that commonly causes otitis media, sinusitis, meningitis, pneumonia and bacteraemia.[1] It is widely known that *S. pneumoniae* is carried asymptomatically within the healthy community, as well as in the vulnerable populations of children, the elderly and those with underlying medical conditions.[2] Carriage rates vary depending on sampling method, sampling site and differences in storage and culture techniques, with carriage rates varying from 2% to over 50%.[3]

In 2003, a World Health Organization (WHO) working group published a report which recommended common methodology when conducting pneumococcal colonisation studies to ensure minimum interstudy method variability. This methodology was recommended as a standard for future studies detecting pneumococcal carriage.[4] One recommendation was the implementation of a storage medium for long-term storage of *S. pneumoniae*.

Various transport and storage media have been used for the transport and storage of *S. pneumoniae* (e.g., Dorset egg medium, Stuart transport medium and Amies transport medium).[5–7] Currently. the most commonly used medium is skimmed milk, tryptone, glucose and glycerin (STGG), which is also recommended by the WHO working group.[1,4]

Antimicrobial resistance in *S. pneumoniae* began to emerge during the late 1970s,[8] and increasingly this pathogen has been isolated with reduced susceptibility to common antibiotics (e.g., penicillin, erythromycin, tetracycline and the newer fluoroquinolone antibiotics). This has led many to believe that the rising number of antibiotic-resistant *S. pneumoniae* strains is a global problem.[9]

Resistance of *S. pneumoniae* to penicillin has been reported widely for a number of years. Penicillin is a member of the β-lactam family of antibiotics and inhibits bacterial growth by interfering with the synthesis of the cell wall, in turn allowing penicillin to exert bactericidal activity against the organism.[10] Penicillin resistance in *S. pneumoniae* is mediated through genetic alterations in a number of the penicillin-binding proteins (PBPs),[11] which are enzymes that perform essential functions in the assembly of the bacterial cell wall.[11]

Evidence suggests that the peptidoglycan structures in the cell wall of penicillin-sensitive strains vary dramatically from penicillin-resistant strains.[11] These alterations correlate solely with a decreased susceptibility to penicillin. Previous studies demonstrate that the cost of penicillin-resistance acquisition for the penicillin-resistant pneumococci competing with its susceptible peers/co-colonisers increases with the number of resistant PBP mutations acquired.[12,13] These studies

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demonstrate that the acquisition of antibiotic resistance has an associated 'fitness' cost to the organism.

In this study, the authors hypothesise that the fitness cost associated with antibiotic resistance via an altered PBP will be positive, enabling the organism to persist/survive better than its antibiotic-susceptible peers. Hence, the aim of this study is to evaluate the survival and recovery of penicillin-sensitive and -resistant strains of *S. pneumoniae* in STGG medium.

Materials and methods

Bacterial strains, culture medium and growth conditions

S. pneumoniae ATCC 49619 and other *S. pneumoniae* strains were used as controls, each showing various sensitivities to penicillin, as described in Table 1. Penicillin susceptibilities were as specified by the Clinical Laboratory and Standards Institute (CLSI). Pneumococci with penicillin minimum inhibitory concentrations (MICs) ≤0.06 μg/mL were considered susceptible.

Cultures were maintained in candle jars on Columbia blood agar (CBA; Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood at 37°C, or stored frozen at –70°C. All strains were confirmed as *S. pneumoniae* by sensitivity to optochin and bile solubility assay.

STGG transport medium

The STGG transport medium was prepared as described previously.[4] Briefly, 2.0 g skimmed milk powder (Oxoid), 3.0 g tryptone soya broth (Oxoid), 0.5 g glucose (Sigma) and 10 mL glycerol (Sigma) were dissolved and mixed with 100 mL distilled water. The solution was aliquoted in 1-mL volumes in screw-capped 5-mL glass Bijou bottles. These were autoclaved at 121°C for 10 min at 15 lb/in^2. Prior to use, the medium was tested for sterility and subsequently stored at 4°C and in the dark until used. The medium was used within three months of preparation.

Culturability at various storage conditions

A loopful of *S. pneumoniae* culture growing on CBA was mixed with 5 mL phosphate buffered saline (PBS) to make an OD600 of approximately 0.4, equivalent to 10^3 colony-forming units (cfu)/mL. From this, samples were inoculated into 1-mL STGG medium to make inoculum densities equivalent to 10^1, 10^3 or 10^5 cfu/mL (this was to ensure that low and high levels of colonisation could be depicted throughout the study).

Table 1. *Streptococcus pneumoniae* strains used in the study.

Strain designation	Penicillin MIC (μg/mL)
ATCC 49619 (control strain)	0.25 (resistant)
2np1	0.016 (sensitive)
0908.1 6 mth	0.125 (resistant)
0509.3 2 mth	0.5 (resistant)
80	<0.03 (sensitive)
27	<0.03 (sensitive)
J5	<0.03 (sensitive)

Sensitivity was defined as an MIC ≤0.06 μg/mL.

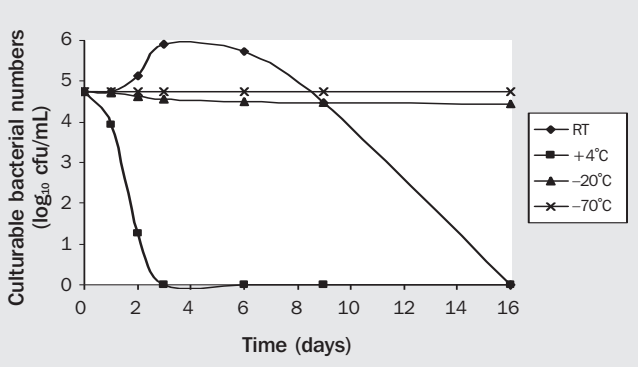

Fig. 1. Survival of the laboratory strain *Streptococcus pneumoniae* ATCC 49619 in STGG medium at different storage temperatures.

A 50 μL sample was plated on CBA using a WASP spiral plater (Don Whitley Scientific) and incubated in candle jars at 37°C for 18–24 h and then counted. This sample was termed day 0 (T0). The remaining volumes of *S. pneumoniae* in STGG were stored at –70°C, –20°C, +4°C and room temperature (RT; average room temperature throughout the course of the experiment was 25.1°C). At various time points from day 1 (T1) to day 16 (T16), the sample, when frozen, would be thawed and a sample plated as described previously.

Following incubation for 18–24 hours, the plates were counted and *S. pneumoniae* numbers recorded. For each *S. pneumoniae* strain tested, mean count was calculated and $P \leq 0.05$ was considered significant, as calculated using the Student's *t*-test.

Results

Optimisation of storage conditions for strain ATCC 49619

Strain ATCC 49619 was used for initial experiments to determine the optimum storage conditions for *S. pneumoniae* in STGG transport medium. Figure 1 shows the mean culturability ($\log_{10}$) of 10^1, 10^3 and 10^5 organisms at different storage temperatures over the course of 16 days. Storage at –70°C allowed culturable bacterial numbers to remain constant throughout the course of the experiment, whereas storage at –20°C showed a decrease of approximately half a log unit during the course of the 16-day period. At a storage temperature of +4°C, culturable bacterial numbers began to decrease within 24 h, quickly declining so that no culturable numbers were detected by day 3. Finally, storage at RT permitted *S. pneumoniae* to proliferate for three days, following which a decrease was observed to the point where no culturable bacteria were detected by day 16.

***Culturability of penicillin-sensitive and penicillin-resistant* S. pneumoniae**

Studies were carried out to determine whether or not *S. pneumoniae* with various MICs to penicillin would influence survival in STGG medium at different storage temperatures. Figure 2 shows mean culturability ($\log_{10}$) of 10^1, 10^3 and 10^5 penicillin-sensitive organisms at different storage temperatures over the course of 16 days.

Culturable *S. pneumoniae* numbers remained constant over the 16-day period while stored at –70°C. Storage at –20°C showed a less than half-log decrease in culturable numbers

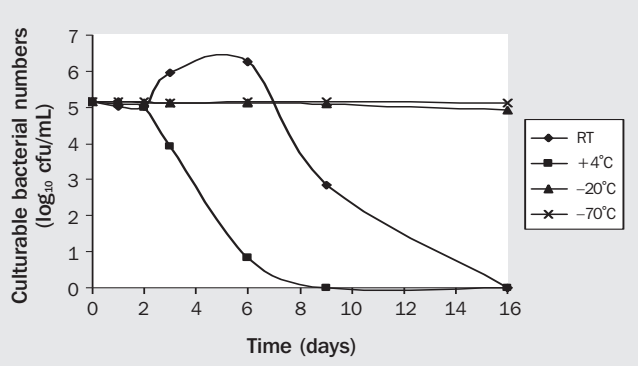

Fig. 2. Survival of penicillin-sensitive strain (22np1) in STGG medium at different storage temperatures.

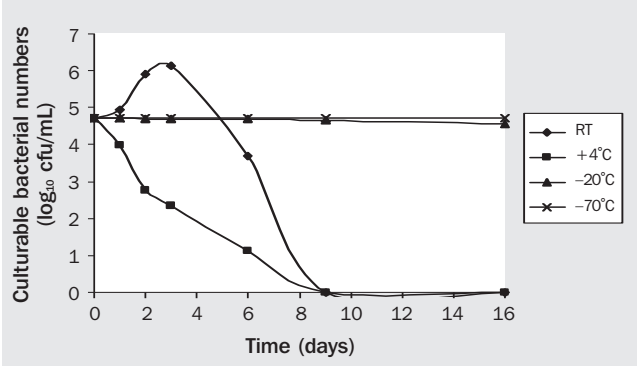

Fig. 3. Survival of penicillin-resistant strain (0908.1 6 mth) in STGG medium at different storage temperatures.

over the 16 days. Storage at +4°C showed that culturable numbers remained relatively constant for up to three days, after which culturability dramatically decreased until day 9 when no culturable organisms were detected. At RT, *S. pneumoniae* numbers remained relatively constant up to day 3, then numbers increased rapidly to a maximum at day 6. Beyond this, numbers decreased dramatically and no culturable organisms were detected by day 16.

Figure 3 shows the mean culturability (log_{10}) of 10^1, 10^3 and 10^5 penicillin-resistant organisms at different storage temperatures over the course of 16 days. Storage at –70°C allowed numbers to remain constant throughout the period of study. Storage at –20°C allowed numbers to remain relatively constant until day 3, beyond which culturability began to decline by less than a half-log to day 16. Storage at +4°C showed a general decline in culturable numbers from day 1 to a point where no organisms could be detected beyond day 9. At RT, bacterial numbers increased rapidly to day 3, following which numbers decreased dramatically and by 16 days no culturability was detected.

The culturability of both penicillin-resistant *S. pneumoniae* strains (0908.1 6 mth and 0509.3 2 mth) was compared to that of the penicillin-sensitive *S. pneumoniae* (22np1), and statistical analysis was carried out. In all cases, no significant difference was found between the penicillin-sensitive and -resistant strains of *S. pneumoniae* at any storage temperature.

Discussion

Many studies have shown that STGG medium is as effective, if not superior, to direct plating alone and has been recommended by a WHO working group for studies investigating pneumococcal carriage.[1,4] Up until now, studies have been conducted using STGG medium in field conditions on nasopharyngeal swabs from individuals.[1,3,14,15] To the authors' knowledge, only one other study has looked at the survival of pure cultures of pneumococci in STGG medium without other nasopharyngeal flora colonisation, which may interfere with survival of the microorganism.[16] In previous studies of pneumococcal carriage, different swabbing sites and isolation methods have shown that *S. pneumoniae* can be recovered after storage in STGG medium.[1,3] In the present study, however, it has been possible to assess the survival of known numbers of pneumococci in a sample.

Previously, STGG medium has been studied in populations with extremely high and dense colonisation rates,[17,18] and in countries with warm climates, such as Australia, Kenya and Tanzania.[14,15,17] The data presented here also show that STGG medium may serve as a growth medium for *S. pneumoniae* in the short-term, particularly in warm climates where storage is at ambient temperature. These experiments initially were carried out during the summer months, when the average ambient temperature was 25.1°C. All strains showed a significant increase in numbers for at least 48 h following initial storage before dropping to undetectable levels. This would suggest that STGG medium may prove to be an effective growth medium for the pneumococcus in the short-term during RT storage. This may explain the high rates of colonisation in warmer regions, as samples stored at ambient temperature for any length of time may go through sample enrichment prior to cold storage.

Previous studies suggest that storage of *S. pneumoniae* in STGG at –70°C is recommended to prevent loss of culturability, storage at –20°C is also considered acceptable but storage at +4°C for up to five days is not ideal.[1] The present study recommends much more stringent conditions for storage of *S. pneumoniae*, particularly where low levels of colonisation may be suspected.

As has been shown in all previous studies, –70°C is not only recommended for long-term storage of samples but for all storage. Storage at –20°C may be acceptable for a few days, although the present study has shown that *S. pneumoniae* numbers can begin to fall within as little as 24 h. Previous studies have shown that storage at –20°C results in survival after 18 months;[16] however, the current work would suggest that low numbers of *S. pneumoniae* may be difficult to detect by culture-based methods after this time. The data presented here would also suggest that in the short-term the culturability of *S. pneumoniae* would be better preserved through storage at RT than at 4°C.

It is widely acknowledged that an individual can carry a number of pneumococcal serotypes at any one time,[1,14] and therefore pneumococci harbouring different levels of antibiotic resistance may also be present at any one time. Antibiotic-resistant pneumococci have emerged as a global problem and the present study considered the difference in culturability of antibiotic-resistant and antibiotic-sensitive strains in STGG medium.

The suggestion that the cell wall structure can vary considerably in penicillin-resistant strains of *S. pneumoniae* compared to penicillin-sensitive strains poses an interesting

question about whether or not resistant strains with a potential difference in cell-wall structure would have different rates of survival in STGG medium. Only minor variations were observed between resistant and sensitive strains to penicillin at any storage temperature. This suggests that *S. pneumoniae* with different antibiotic resistance profiles are not being selected preferentially through storage in STGG medium.

It is possible that the presence of the total nasopharyngeal flora may influence the pneumococcus and may provide better conditions for survival, in particular at less favourable storage temperatures. A small pilot study carried out combining approximately 10^3 *S. pneumoniae* ATCC 49619 organisms with an equal number of the viridans species *S. sanguinis* NCTC 7863 in STGG medium and subsequent storage at –70°C, –20°C and +4°C showed similar results to survival of *S. pneumoniae* in STGG medium alone (data not shown).

Briefly, *S. pneumoniae* numbers remained constant through storage at –70°C, a 50% decrease in *S. pneumoniae* was detected following 48-h storage at –20°C and a 90% decrease in numbers was detected following 48-h storage at +4°C. Spiking an oral rinse of sterile PBS with 10^3 *S. pneumoniae* organisms combined with an equal volume of STGG medium, and storage at the three temperatures, yielded similar results (data not shown). Although the current study concentrated on determining the survival of *S. pneumoniae* alone in STGG medium and did not investigate in any depth the influence of accompanying flora on survival of the organism, preliminary data would suggest that accompanying flora has little or no effect on *S. pneumoniae* survival.

In conclusion, this study has confirmed that storage of pneumococci at –70°C remains the best method to ensure recovery of culturable organisms. While storage may be acceptable in the short-term at –20°C, storage at +4°C is not recommended. Furthermore, STGG medium at RT has proved to be a growth medium for pneumococci in the short-term. The survival of strains resistant to penicillin was comparable with that of sensitive strains, thus no bias in the recovery of antibiotic-resistant strains over antibiotic-sensitive strains was afforded by storage in STGG medium. □

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