Survival of Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis Frozen in Skim Milk-Tryptone-Glucose-Glycerol Medium

Tarja Kaijalainen,1* Esa Ruokokoski, 2 Pentti Ukkonen,2 and Elja Herva1

KTL, National Public Health Institute, Oulu,1 and Helsinki,2 Finland

Received 17 May 2002/Returned for modification 29 September 2002/Accepted 11 October 2003

In STGG (skim milk, tryptone, glucose, glycerol) medium at −80°C, Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis isolates survived for at least 3 years, and the same species have survived in nasopharyngeal swabs for at least 1.5 years. At −20°C, S. pneumoniae and M. catarrhalis survived for 1.5 years, but H. influenzae survived for only 2 months.

Pneumococcus (Streptococcus pneumoniae), Haemophilus influenzae, and Moraxella catarrhalis are part of normal nasopharyngeal (NP) microbial flora and are important causes of acute otitis media in children (4, 7). Pneumococcus and H. influenzae serotype b are also leading causes of serious infections, such as pneumonia, meningitis, and bacteremia in children. Vaccines against pneumococcus and H. influenzae serotype b are serotype specific, and the prevalence of the different types in different geographic areas should be known. Reliable methods are needed for the collection, transport, and storage of both NP samples and bacterial isolates from study sites to specialized laboratories even in developing country settings. STGG (skim milk, tryptone, glucose, glycerol) medium (1, 2) has been used successfully for storage at −70 to −80°C of isolates of S. pneumoniae and H. influenzae (2) and of NP swabs for detection of the same bacteria (3, 4).

In this study, we used STGG medium (1, 2) as described by O’Brien and coworkers (4). One milliliter of STGG medium was distributed in screw-cap 3.6-ml tubes, autoclaved at 121°C, and kept at 4°C. For viability subcultures, we used sheep blood agar (BA) without and with gentamicin (5 μg/ml) (GBA) plates for S. pneumoniae, enriched chocolate agar (CA) plates for H. influenzae, and BA plates for M. catarrhalis (6, 7).

We first tested survival of S. pneumoniae (ATCC 6305), M. catarrhalis (ATCC 25238), and H. influenzae (a clinical isolate) isolates in 1 ml of STGG in 3.6-ml screw-cap tubes. For S. pneumoniae, three STGG tubes were prepared for each temperature (−20°C and −80°C) by inoculation with (i) five colonies lightly touched with a 1-μl loop, (ii) a 1-μl loopful, or (iii) plenty of growth. For H. influenzae and for M. catarrhalis, one tube for each temperature was inoculated with plenty of growth. One further tube for each temperature was inoculated with plenty of all the three bacteria. The tubes were kept at −20 and −80°C, and subcultures for viability testing were performed 18 times over a period of 3 years at variable time intervals (see Fig. 1 and Results).

For subculturing, the STGG tubes were taken from the freezers and kept on the bench (at room temperature) until thawed, the contents were mixed by vortexing, and 10 μl of each bacterial suspension was streaked on the appropriate plate. The tubes were transferred immediately into freezers. The plates were incubated at 37°C for 18 to 24 h. Bacterial colonies were counted, and the results of this process were expressed as follows: no growth (<1 colony), 1 to 20 colonies, 21 to 100 colonies, and >100 colonies.

The S. pneumoniae and M. catarrhalis isolates survived at −80°C without a change in the number of colonies throughout the follow-up period of 3 years and 18 times following the described process. The number of H. influenzae colonies decreased after 15 months (12 repeated processes). The number of colonies of all bacteria decreased at −20°C, and only S. pneumoniae survived the whole period (Fig. 1). The amount of bacterial growth inoculated, when testing for S. pneumoniae only, did not have any influence on recovery (data not shown). Also, in the mixture of the three species, S. pneumoniae and M. catarrhalis survived well at −80°C for 3 years with 18 processes and H. influenzae, with a decreased number of colonies, for up to 2 years. The number of colonies decreased earlier at −20°C than at −80°C, and only M. catarrhalis survived the whole period (Fig. 2).

We also studied the survival of S. pneumoniae, H. influenzae, and M. catarrhalis in NP samples obtained from 10- to 24-month-old children participating in a study on the prevention of acute otitis media (7). NP secretions were collected by swabs and stored in STGG at −70 to −80°C and cultured for the bacteria as described earlier (6, 7). One and a half years later, 100 consecutive NP swabs were chosen for repeat cultures, performed similarly to the original cultures in the same laboratory and following the same written instructions. The results were recorded, and after this, the results of the original cultures were retrieved from the study files by the data manager for comparison.

Table 1 shows the growth of S. pneumoniae, H. influenzae, and M. catarrhalis from NP swabs in STGG medium as cultured after 1.5 years of storage at −80°C, compared to original cultures. The results of the repeat and the original cultures were almost identical.

Earlier studies have demonstrated the good performance of STGG medium in deep-freeze storage of S. pneumoniae and other bacterial species (2–4). S. pneumoniae has been shown to

* Corresponding author. Mailing address: KTL, National Public Health Institute, POB 310, FIN-90101 Oulu, Finland. Phone: 358-8 5376249. Fax: 358-8-5376251. E-mail: tarja.kaijalainen@ktl.fi.
FIG. 1. Numbers of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* colonies after storage in STGG medium at -20 and -80°C.

FIG. 2. Numbers of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* colonies after storage as a mixture in STGG medium at -20 and -80°C. The symbols of bacterial species are grouped by temperature: top row, *S. pneumoniae*; middle row, *H. influenzae*; and bottom row, *M. catarrhalis*. The symbols of bacterial species are the same as in Fig. 1: left row, *S. pneumoniae*; middle row, *H. influenzae*; and right row, *M. catarrhalis*. The symbols of bacterial species are grouped by temperature:
remain viable in STGG medium at both −20 and −70°C for at least 9 weeks (4). When preserved in various other media (rabbit blood, sheep blood, skim milk, glycerol-chocolate broth), S. pneumoniae also remains viable for a few months at both −20 and −70°C, but for more than 1 year only at −70°C (5).

Our results confirm and extend these findings by demonstrating that STGG medium is excellent for storage of S. pneumoniae and also H. influenzae and M. catarrhalis at −80°C for long periods. In addition, it can be used for storage at −20°C for shorter periods, even under conditions with a less-than-ideal supply of electricity. Because the amount of pneumococcal growth stored in STGG tubes did not have a clear effect on recovery, it is not necessary to subculture to obtain plenty of growth for storage.

In their detailed study, O’Brien and coworkers (4) showed that collection, transport, and storage of NP swabs for S. pneumoniae in STGG medium has several benefits, including enhanced recovery, long-term storage of original NP specimens at −70°C without any loss of CFU, and assay of NP specimens in a batch form. We used STGG medium for collection and storage at −80°C of NP swabs from healthy Finnish children (7) and found colonization rates of S. pneumoniae and H. influenzae similar to those in another study in Finland, where direct plating was used (6). On the basis of these experiences and the results of the present study, we fully agree with the conclusions of O’Brien and coworkers (4), extending them further to two other respiratory tract pathogens, H. influenzae and M. catarrhalis.

We are grateful to P. Helena Mäkelä and Heljä-Marja Surcel for helpful comments. We also thank Katriina Autio, Tiina Halunen, Aili Hökkä, Eeva-Liisa Korhonen, and Virpi Määttä for excellent technical assistance.

**REFERENCES**


