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Viability of respiratory pathogens cultured from nasopharyngeal swabs stored for up to 12 years at −70 °C in skim milk tryptone glucose glycerol broth

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ABSTRACT

Nasopharyngeal carriage studies are needed to monitor changes in important bacterial pathogens in response to vaccination and antibiotics. The ability to store original specimens frozen in skim milk tryptone glucose glycerol broth (STGGB) allows additional studies to be conducted without the need for further expensive field collection. Although sub-cultured isolates remain viable in this medium for many years, limited data are available to indicate viability of relatively low numbers of organisms present in nasopharyngeal specimens stored frozen over long periods of time. We conducted several studies whereby swabs stored in STGGB at −70 °C for up to 12 years were thawed and aliquots cultured. Recovery of *Streptococcus pneumoniae* (72% positive from 269 swabs), *Haemophilus influenzae* (62% from 214) and *Moraxella catarrhalis* (81% from 162) was not significantly different from the original cultures: 69% (Risk Difference [RD] 3.0, 95% Confidence Interval [CI] −4.7, 10.7), 66% (RD −4.7, 95% CI −13.8, 4.4) and 78% (RD 3.1, 95% CI −5.7, 11.9) positive respectively. There was no trend in recovery from swabs stored for increasing lengths of time. We conclude that studies which rely on the viability of these respiratory pathogens can be conducted using original swabs stored at −70 °C for at least 12 years.

1. Introduction

Skim milk tryptone glucose glycerol broth (STGGB) has several advantages over direct plating, including long-term storage of the original nasopharyngeal (NP) swab, the opportunity to inoculate multiple plates from the original sample, homogeneous dispersion of the specimen, and the ability to quantify the growth of organisms (O'Brien and Nohynek, 2003). The use of standard aliquots from the medium in which the swab is stored for plating, rather than the swab itself, also means the swab cannot be 'contaminated' by antibiotics in selective media and so remains a 'pristine' representation of the original specimen. While originally developed for frozen storage of bacterial isolates (Gibson and Khoury, 1986), and then used for NP swabs, STGGB (prepared as a concentrate) can also be used to store other specimens such as NP aspirates (Moore et al., 2010), sputum and bronchoalveolar lavage fluid (Hare et al., 2010b). The ability to store original specimens frozen in STGGB allows additional studies to be conducted (provided that appropriate consent has been obtained from study participants) without the need for further expensive field collection (Hare et al., 2010a). Additional hypotheses can be tested; for example, retrospective monitoring of the emergence of important new pathogens and study of carriage dynamics. As scientific technologies advance, stored specimens can be analyzed with greater precision or analyses not previously possible may be undertaken. Recently our laboratory and others have found that PCR detection of respiratory bacteria and viruses (Moore et al., 2010; Binks et al., 2011), and ELISA assays of certain immunological markers (unpublished data), are not inhibited by specimen storage in STGGB. Others have shown that sub-cultured isolates remain viable in this medium for many years (Kaijalainen et al., 2004). We now provide evidence that relatively low numbers of organisms present in NP swabs remain viable following frozen storage over long periods of time.

2. Methods

Four follow-up studies were undertaken at different times using swabs from four original studies (Bailie et al., 2003; Leach et al., 1994; Leach et al., 2008; Mackenzie et al., 2009) and a pilot trial, in six different trial combinations (Table 1). The first follow-up study specifically evaluated recovery of pneumococci from stored swabs, while the others took advantage of swabs being thawed for other purposes (Binks et al., 2011; Smith-Vaughan et al., 2006; Smith-Vaughan et al., 2008). Swabs had previously been thawed a maximum of 3 times. In all trials, NP or nasal swabs stored at −70 °C (till the early 2000s, and subsequently −80 °C) in STGGB for a variable number of years (Table 2) were thawed, vortexed and 10 μL aliquots plated on selective media. In the first trial, aliquots were plated on 5% horse blood agar (HBA) and HBA containing colisten and nalidixic acid (HBA–CNA, Oxoid Australia) only. In subsequent trials, aliquots were plated on HBA, chocolate agar, HBA–CNA and bacitracin vancomycin clindamycin chocolate agar for recovery

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Table 1
Study information.

Original study name	NH and PAT studies	COMIT	CHIPS	Natural history	COMIT	PRIORiTI
Years	1992–1995	1996–2001	2001	1992–1993	1996–2001	2001–2004
Description	Natural history study (1992–1993, see column five)[a] and Pilot Antibiotic Trial (1994–1995)[b]	RCT of amoxicillin versus placebo for OM with effusion in Indigenous children[c]	Longitudinal child care hygiene intervention study in mostly non-Indigenous children[d]	Longitudinal birth cohort study of NP carriage and onset of OM in Indigenous infants[a]	RCT of amoxicillin versus placebo for OM with effusion in Indigenous children[c]	Longitudinal study of NP carriage and OM in Indigenous infants receiving 7-valent PCV[e]
Follow-up study	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
Year	1998	2003		2004	2008	
Description	Study to evaluate recovery of pneumococci from stored swabs	Retrospective study of nasal bacterial load and suppurative OM in Indigenous and non-Indigenous children[f]		Retrospective study of nasal bacterial load and onset of OM in Indigenous infants[g]	Retrospective study of respiratory bacteria and viruses in NP swabs from Indigenous children with and without acute OM[h]	

NP, nasopharyngeal; OM, otitis media; PCV, pneumococcal conjugate vaccine; RCT, randomised controlled trial.
[a] Leach et al., 1994.
[b] Cotrimoxazole versus amoxicillin crossover study for OM in Indigenous children, unpublished data.
[c] Leach et al., 2008.
[d] Bailie et al., 2003.
[e] Mackenzie et al., 2009.
[f] Smith-Vaughan et al., 2006.
[g] Smith-Vaughan et al., 2008.
[h] Binks et al., 2011.

of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*. In one of the original studies (Bailie et al., 2003), non-selective plates were not used therefore *M. catarrhalis* was not recovered. Plates were incubated overnight at 37 °C and 5% CO_2 and read blinded to the original results. Semi-quantitative colony counts were recorded as previously described (Smith-Vaughan et al., 2006). Where counts from two or more plates were made for the same organism, the higher score was recorded (as in the original studies).

Pneumococcal identification was confirmed by colony morphology, susceptibility to optochin and a positive quellung reaction with pneumococcal antisera (Statens Serum Institut, Denmark). *H. influenzae* was confirmed by colony morphology and requirement for X and V growth factors. *M. catarrhalis* was confirmed by colony morphology, oxidase positivity and Gram stain morphology. The same culture and identification methods were used for the original and follow-up studies.

Stata version 10 was used for all analyses. Risk differences (RD) were estimated from 2×2 tables with confidence intervals (CI) calculated by the exact binomial method. Dichotomous agreement for positive and negative swabs was assessed using the kappa statistic. Categorical agreement for 7 categories of semi-quantitative growth from positive swabs and one category of negative swab was assessed by the weighted kappa statistic.

3. Results

Recovery of respiratory pathogens from swabs selected for additional analyses was compared with the original results. Carriage data were obtained for *S. pneumoniae* from 269 swabs (6 trials), for *H. influenzae* from 214 swabs (5 trials) and for *M. catarrhalis* from 162 swabs (4 trials) as shown in Table 2. Total recovery of *S. pneumoniae* (72%), *H. influenzae* (62%) and *M. catarrhalis* (81%) after long-term frozen storage was not significantly different from the original cultures: 69% (RD 3.0, 95% CI −4.7, 10.7), 66% (RD −4.7, 95% CI −13.8, 4.4) and 78% (RD 3.1, 95% CI −5.7, 11.9) positive respectively. Recovery of *S. pneumoniae* was actually higher after storage in 4 of 6 trials, lower in one and the same in one. Similarly, recovery of *M. catarrhalis* was higher after storage in 3 of 4 trials where it was recorded at both times. However recovery of *H. influenzae* after storage was lower in 4 of 5 trials. None of the differences from individual studies was statistically significant (data not shown), and there was no apparent trend in recovery over time (Fig. 1).

Numbers of swabs originally positive and subsequently negative, and vice versa, for the three pathogens are shown in Table 3A, B and C. Sensitivities of recovery after storage at −70 °C in STGGB for up to 12 years were 97%, 89% and 96% for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* respectively and specificities were 83%, 92% and 72%

Table 2
Carriage rates detected in original studies and after long-term storage of nasopharyngeal swabs in STGGB at −70 °C.

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
Median age in days (range)	na[a]	723 (543 to 1053)	885 (562 to 1095)	41 (2 to 152)	219 (58 to 505)	172 (44 to 578)
Years stored	3 to 6	2 to 6	2	11 or 12	7 to 12	4 to 6
Number of swabs thawed	55	51	52	36	49	26
Streptococcus pneumoniae positive						
Originally	45 (82%)	43 (84%)	25 (48%)	17 (47%)	41 (84%)	15 (58%)
After storage	48 (87%)	46 (90%)	26 (50%)	17 (47%)	40 (82%)	17 (65%)
Haemophilus influenzae positive						
Originally	na[a]	40 (78%)	25 (48%)	17 (47%)	39 (80%)	21 (81%)
After storage	Not done	36 (71%)	21 (40%)	15 (42%)	42 (86%)	18 (69%)
Moraxella catarrhalis positive						
Originally	na[a]	45 (88%)	Not done	16 (44%)	43 (88%)	22 (85%)
After storage	Not done	49 (96%)	na[a]	14 (39%)	44 (90%)	24 (92%)

[a] na, not available.