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Note

Haemophilus influenzae isolates survive for up to 20 years at −70 °C in skim milk tryptone glucose glycerol broth (STGGB) if thawing is avoided during re-culture

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ABSTRACT

Haemophilus influenzae remains a major cause of disease worldwide requiring continued study. Recently, isolates of *Streptococcus pneumoniae* and *Moraxella catarrhalis*, but not *H. influenzae*, were reported to survive long-term ultra-freeze storage in STGGB. We show that nontypeable *H. influenzae* isolates survive for up to 20 years when thawing is avoided.

Diseases associated with *Haemophilus influenzae* represent a major health burden for young children and adults worldwide. Although invasive disease due to *H. influenzae* type b (Hib) has been substantially reduced since the introduction of Hib conjugate vaccines, disease due to Hib persists and replacement with non-type b *H. influenzae* strains has occurred (Bruce et al., 2008; Menzies et al., 2015). Furthermore, nontypeable *H. influenzae* (NTHi) is the major pathogen associated with chronic middle ear and lung disease, with particularly high rates in Indigenous children (Hare et al., 2010; Smith-Vaughan et al., 2013). NTHi is also the major pathogen responsible for respiratory tract infections in adults with chronic obstructive pulmonary disease, and an effective vaccine is needed (Murphy, 2015).

Storage of bacterial isolates recovered from clinical or research specimens enables subsequent testing should new questions arise or new technologies be developed. Such studies may investigate virulence factors, antibiotic resistance determinants or potential vaccine antigens. Skim milk tryptone glucose glycerol broth (STGGB) was originally developed for ultra-freeze storage of bacterial isolates (Gibson and Khoury, 1986). Our group was the first to use STGGB for the storage of nasopharyngeal swabs (NPS) and swabs of ear discharge in studies of carriage and otitis media (Leach, 1996). STGGB has proved invaluable for the storage of NPS and other specimens harboring respiratory bacteria, and remains the medium of choice for pneumococcal carriage studies (Satzke et al., 2013).

Previously we showed that *Streptococcus pneumoniae*, *H. influenzae* and *Moraxella catarrhalis* remain viable in NPS stored for up to 12 years at −70 °C in STGGB (Hare et al., 2011). Similar results were recently published with all three species surviving in NPS stored at −70 °C for 11 years (Kaijalainen and Palmu, 2015). All three pathogens had been shown to remain viable as isolates in STGGB at −70 °C for 3 years (Kaijalainen et al., 2004). In the follow-up publication, *S. pneumoniae* and *M. catarrhalis* isolates were found to survive for up to 12.5 years, whereas *H. influenzae* isolates did not survive even for 4 years (Kaijalainen and Palmu, 2015). We report our isolate survival data in order to reassure researchers that loss of *H. influenzae* viability can be avoided.

Isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* from our studies of carriage and otitis media since 1992 (Leach et al., 1994), and from our lung studies in children since 2007 (Hare et al., 2010), are stored in STGGB at −70 °C (−80 °C since the early 2000s). Periodically, isolates have been regrown for further studies. For example, pneumococcal isolates (from NPS) stored in 1996–8 and 2002–4 were regrown in 2008 to differentiate serotype 6A from 6C (Hare et al., 2009), demonstrating *S. pneumoniae* isolate survival for up to 12 years. Similarly, phenotypic NTHi isolates (from NPS, oropharyngeal swabs (OPS) and bronchoalveolar lavage (BAL) specimens) stored in 2007–10 were regrown in 2011 for *hpd* PCR (Binks et al., 2012) to differentiate *H. influenzae* from the closely-related primarily commensal *H. haemolyticus* (Hare et al., 2012). This *hpd* PCR analysis confirmed most phenotypic NTHi isolates from NPS and BAL as *H. influenzae* and demonstrated survival of *H. influenzae* isolates in STGGB at −80 °C for up to 4 years (Table 1).

Additional phenotypic NTHi isolates from our lung studies were subsequently regrown for *hpd* PCR, whole genome sequencing (Smith-Vaughan et al., 2014) and *fucP* PCR (Price et al., 2015), including several isolates that had previously tested negative using *hpd* PCR (Table 1). These retrospective analyses of isolates stored in STGGB at −80 °C for

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Table 1
Viability of stored phenotypic nontypeable *Haemophilus influenzae* isolates and results of *hpd* and *fucP* PCR testing.

Year stored	Years in storage	Isolates tested[a]	*hpd* PCR positive	Years in storage	Isolates tested[a]	*fucP* PCR positive
Nasopharyngeal swab isolates						
1995				20	3	3
2007	4	9	9			
2008	3	23	21			
2009	2	25	19	5	1	1
2010	1	20	18			
2011	2	9	8	3	1	1
2012	1	9	9	2	1	1
2013	0	16	16	1	19	18
2014				0	17	17
Total		111	100 (90%)		42	41 (98%)
Oropharyngeal swab isolates						
1995				20	4	2
2008	3	13	5			
2009	2	28	9	5	1	1
2010	1	24	8	4	1	0
2011	2	7	3	3	2	0
2012	1	9	8	2	1	0
2013	0	10	5	1	23	12
2014				0	14	5
Total		91	38 (42%)		46	20 (43%)
Bronchoalveolar lavage isolates						
2007	4	19	18			
2008	3	22	20	6	1	1
2009	2	23	16	5	1	1
2010	1	8	8			
2011	2	4	4	3	1	0
2012	1	11	7	2	4	2
2013	0	2	2	1	12	12
2014				0	5	5
Total		89	75 (84%)		24	21 (88%)

[a] All isolates selected for testing were successfully regrown.

up to 6 years allowed us to confirm the presence or absence of important vaccine antigens and to discriminate pathogenic NTHi from the commensal *H. haemolyticus* (*fucP* negative).

We then located seven phenotypic NTHi isolates stored in 1995 (Leach et al., 1997; Shelby-James et al., 2002); 5 isolates (3 NPS, 2 OPS) were confirmed as *H. influenzae* by *fucP* PCR (Table 1) and two OPS isolates were re-classified as *H. haemolyticus*. No isolate selected for testing in the above-mentioned studies failed to re-grow. Therefore we have demonstrated that both *H. influenzae* and *H. haemolyticus* isolates can survive ultra-freeze storage for at least 20 years.

Kaijalainen and Palmu (2015) reported that isolates were thawed and refrozen throughout the study period after 4, 8.5, 11.5 and 12.5 years of storage. In contrast, we do not thaw our isolates. Cryovials of each isolate in STGGB are kept briefly on ice while a scraping from the top of each vial is taken by 'drilling' into the frozen media using a disposable 10 μL loop. Using this method, isolates can be regrown and tested repeatedly (as described above) without compromising their long-term viability. Our freezers are connected to an uninterruptible power supply and alarm system, and a backup freezer is kept at −80 °C in case of freezer breakdown, to ensure that specimens and isolates are kept frozen. An alternative strategy is to take an aliquot from each original specimen after vortexing for storage in a separate freezer (Satzke et al., 2013). Isolates can similarly be aliquoted into two (or more) cryovials for storage in separate freezers to avoid loss if one freezer breaks down. *H. influenzae* isolates frozen in STGGB can also be stored in liquid nitrogen with no impact on long-term viability (SG Tristram, personal communication).

Although Kaijalainen and Palmu (2015) found that *S. pneumoniae* and *M. catarrhalis* remained viable as isolates despite repeated thawing and freezing, we suggest that the practice of repeated freeze–thaw compromised *H. influenzae* viability. We recommend several strategies to maximise *H. influenzae* survival in long-term ultra-freeze storage, thus enabling studies of virulence factors, antibiotic resistance determinants and vaccine candidates that rely on viable stored isolates.

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