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## Optimum Condition for Storage of Sputum from Patients with Bronchiectasis Infected with Haemophilus Influenza

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### Abstract

**Background:** The optimum storage condition is not known for the isolation of *H. influenzae* in bronchiectasis patient sputum samples, the most common pathogen among bronchiectasis patients. The aim of the study was to assess the optimum sputum storage conditions for the isolation of *Haemophilus influenzae* from microbiological culture in patients with bronchiectasis.

**Method and Findings:** 20 stable bronchiectasis patients were recruited. The first three patients were used to assess reproducibility. 11 patient sputum samples were then collected and then stored for 2, 4, 6, 24 and 48 hours at room temperature and for 24 and 48 hours at 4°C, -20°C or -70°C before processing. The final 6 patient sputum samples were stored in 25% glycerol, 50% skimmed milk and neat at 4°C, -20°C and -70°C for 7 days before processing. Bacteria were identified and median log<sub>10</sub> bacterial density (c.f.u./ml<sup>-1</sup>) and viable rates were calculated. There was good reproducibility with the coefficient of variation varying between 2-9%. The median baseline density of *H. influenzae* at room temperature was 1.2×10<sup>8</sup> c.f.u. ml<sup>-1</sup>. There was no significant difference in the density of *H. influenzae* cultured from the samples stored at room temperature and 4°C for any of the time points up to 48 h (room temperature: 48 h, 1.0×10<sup>8</sup> c.f.u. ml<sup>-1</sup> and at 4°C: 48 h, 6.5×10<sup>7</sup>c.f.u. ml<sup>-1</sup>). Viable rates were the highest in samples left at room temperature for 48 h (81%). After 7 days of storage, only the sample stored at -70°C in glycerol (5.2×10<sup>7</sup>c.f.u. ml<sup>-1</sup>) showed no significant difference in the density of *H. influenzae* compared to processing right after collection (1.2×10<sup>8</sup>c.f.u. ml<sup>-1</sup>) and viable rates of 91%.

**Conclusion:** The optimum conditions for sputum storage and processing for the isolation of *H. influenzae* from bronchiectasis patients are the storage at room

temperature for up to 48h and storage at -70°C in glycerol for up to 7 days.

**Keywords:** Bronchiectasis; Sputum processing; *Haemophilus influenzae*; Temperature; Medium

### Introduction

Bronchiectasis is a common chronic debilitating respiratory condition with permanent airway damage that leads to patients having recurrent cough, sputum production and recurrent chest infections. Almost two thirds of bronchiectasis patients are chronically colonized with bacterial pathogens [1]. Assessing sputum microbiology is important in the management of patients, and in practice, the sputum sample is processed as soon as possible. However, we know from experience that there can be a long time between sample collection and processing. Long-term storage of specimens can also offer the opportunity to do future research on them [2], which makes the storage of samples important, as it may have a direct influence on the recovery of viable organisms. Nearly 55% of bronchiectasis patients are chronically colonized with *H. influenzae*, which is the most common isolated species from sputum [1,3]. *H. influenzae* was first described in 1892 by Richard Pfeiffer. It usually colonizes the upper airways, but it is also frequently isolated from the lower airways of patients with chronic obstructive pulmonary disease or bronchiectasis [4]. To the author's best knowledge, there have been no studies addressing the optimum storage conditions for the isolation of *H. influenzae* in sputum samples. This study aims to identify the optimum time, temperature and medium for the storage of samples from patients with bronchiectasis chronically infected with *H. influenzae*.

## Methods

### Patients

20 clinically stable patients with bronchiectasis and a history of chronic *H. influenzae* infection were recruited from the Bronchiectasis Clinic at the Royal Infirmary of Edinburgh in Scotland, UK. The study received ethical approval from the Lothian research ethics committee and NHS Lothian R+D approval.

In this study we only included patients with bronchiectasis confirmed by chest CT according to the features described by Naidich et al. [5] and clinical symptoms including chronic cough and sputum production when clinically stable. Only patients that cultured *H. influenzae* alone were included in the study. We excluded current or ex-smokers who stopped smoking less than one year ago or ex-smokers with a history of more than 20 pack-years of smoking. We also excluded patients who were on long-term oral or inhaled antibiotics longer than 28 days before the study.

Out of the 20 patients, three were recruited to assess reproducibility for quantitative microbiology. For the

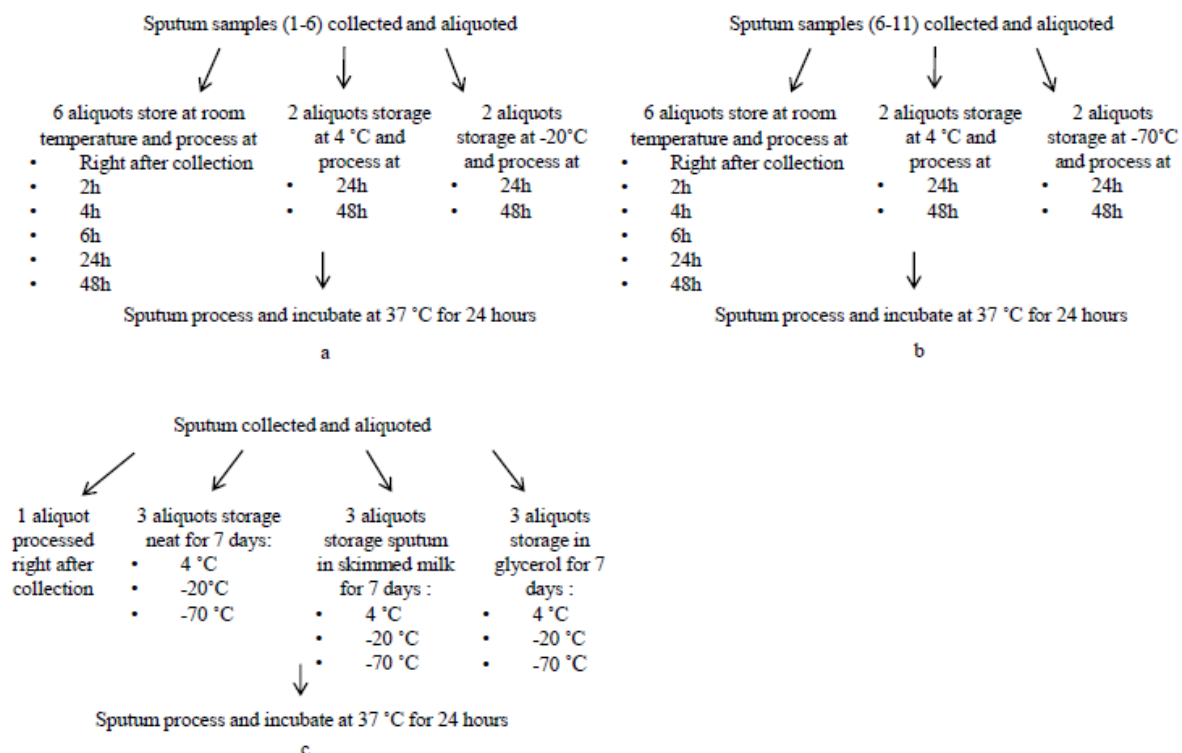
experiments three samples were plated in triplicate. 17 patients were recruited for the sputum storage experiments.

### Sputum collection

All patients were taught chest clearance techniques using the active cycle breathing technique. Patients were asked to provide a spontaneous sputum sample into a sterile container at the Royal Infirmary of Edinburgh to allow immediate processing.

### Sputum processing and storage

Sputum was collected and each sputum sample was immediately aliquoted into separate sterile containers. 11 samples were stored short term (2, 4, 6, 24 and 48 hours) at room temperature, 4°C, -20°C and -70°C (Figures 1A and 1B). Samples 1-5 were each aliquoted into 10 specimens, which were divided as follows: one specimen was processed immediately after collection, five specimens were stored at room temperature (23°C) for 2, 4, 6, 24 and 48 hours, two specimens were stored at 4°C for 24 and 48 h and two specimens were stored at -20°C for 24 and 48 h.



**Figure1:** Method of sputum sample short term storage and processing.

Sample 6 was aliquoted into 12 specimens, which were divided as follows: one specimen was processed immediately after collection, five specimens were stored at room temperature (23°C) for 2, 4, 6, 24 and 48 hours, two specimens were stored at 4°C for 24 and 48 h, two specimens were stored at -20°C for 24 and 48 h and two specimens were stored at -70°C

for 24 and 48 hours. Samples 7-11 were each aliquoted into 10 specimens, which were divided as follows: one specimen was processed immediately after collection, five specimens were stored at room temperature (23°C) for 2, 4, 6, 24 and 48 hours, two specimens were stored at 4°C for 24 and 48 h and two specimens were stored at -70°C for 24 and 48 hours.

6 sputum samples were each aliquoted into 10 specimens and stored long term (7 days). 1 specimen from each sample was processed immediately after collection, 3 specimens from each sample were stored with 25% glycerol at 4°C, -20°C and -70°C. 3 specimens from each sample were stored with 50% skimmed milk at 4°C, -20°C and -70°C. 3 specimens from each sample were stored neat at 4°C, -20°C and -70°C (Figure 1C).

### Sputum processing

At the designated time points for processing, samples were prepared for qualitative and quantitative bacteriology as described by Pye et al. [6]. Briefly, sputum was homogenized and liquefied using an equal volume of Sputolysin (Calbiochem Ltd), and serially diluted using sterile 0.9% saline to achieve dilution factors of  $10^{-1}$  to  $10^{-4}$ . Chocolate blood agar plates were inoculated with  $100 \mu\text{l}$   $10^{-1}$  and  $10^{-4}$  sample, and then incubated in an aerobic atmosphere enriched with 5-10% carbon dioxide at 37°C for 48 hours. Colonies were then counted to determine the sputum *H. influenzae* density expressed as  $\log_{10}$  c.f.u.  $\text{ml}^{-1}$ , in addition the viable rate was calculated by comparing the colony numbers from the samples processed immediately after collection to all other time points, temperatures and storage media (observed bacterial load/ original bacterial load $\times 100$ ).

### Identification of *H. influenzae*

The identification of *H. influenzae* was based on the requirement of both X and V factor on blood agar.

### Statistics

Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software). The data are presented as means with their standard deviations for normally distributed data and medians with their interquartile ranges for data that were not normally distributed. A paired t-test or Wilcoxon analysis for two groups was performed. An ANOVA was used to compare multiple groups. We use the coefficient of variation to assess internal consistency. A two-tailed P value of 0.05 was considered statistically significant.

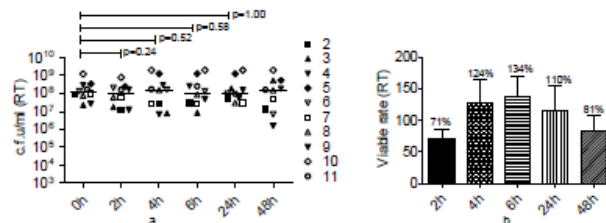
### Results

Out of the 20 patients, 3 were recruited to assess reproducibility for quantitative microbiology (2 females and 1 male, aged 67, 79 and 35 years). 17 patients (9 females and 8 males) with an average age of 65 years old (range 22 to 84 years) were recruited for the sputum storage experiments. On computed tomography, there were a median of 4 (2.75 to 5) bronchiectatic lobes involved.

In the three experiments to assess reproducibility for quantitative microbiology, the coefficients of variation were 4%, 9% and 2% (Figure 2).

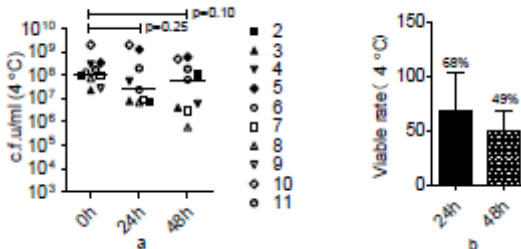
All samples stored at any conditions grew *H. influenzae*. There was no significant reduction in *H. influenzae* density between specimens processed immediately after collection and the specimens stored at room temperature from 2 to 48 hours

(Figure 2A). The average viable rate for the sample processed at 2 hours was 71%, at 4 hours was 124% and at 6 hours was 134%. After storage at room temperature for 24 and 48 hours, the viable rates was 110% and 81% respectively (Figure 2A).



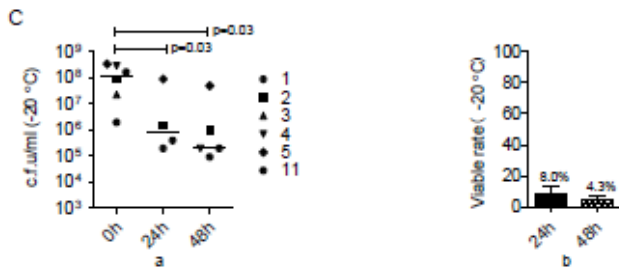
**Figure 2:** The different dot shapes represents different samples. The solid line represents the median value, The 0 h represents process immediately after collection, The dot hasn't been show is 0. 4 represents sputum stored at 4°C, -20 represents sputum stored at -20°C, -70 represents sputum stored at -70°C- A (a), Sputum bacterial density of specimens processed right after collection and after storage 2, 4, 6, 24 and 48 hours at room temperature-A (b), the viable rates of samples stored in different conditions.

There was no significant decrease in *H. influenzae* density between the specimen processed right after collection and specimens stored at 4°C for 24 and 48 hours (Figure 2B). The viable rates for specimens processed after 24 and 48 hours was 63% and 46% respectively (Figure 2B).



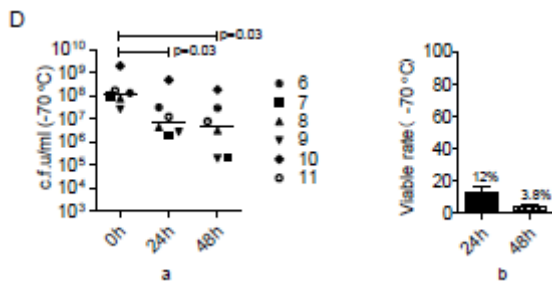
**Figure 2B:** (a), Sputum bacterial density of specimens processed right after collection and after storage 24 and 48 hours at 4°C (a), B (b), the viable rates of samples stored in different conditions.

There was a significant reduction in *H. influenzae* density between the specimen processed right after collection and specimens stored at -20°C for 24 and 48 hours (Figure 2C). The viable rates for specimens processed after 24 and 48 hours was 8.0% and 4.3% respectively (Figure 2C).



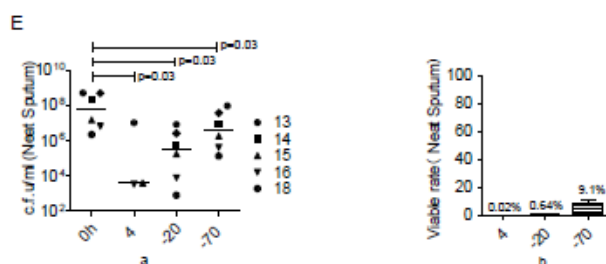
**Figure 2C:** (a), Sputum bacterial density of specimens processed right after collection and after storage 24 and 48 hours at  $-20^{\circ}\text{C}$ , C (b), the viable rates of samples stored in different conditions.

There was a significant reduction in *H. influenzae* density between the specimen processed right after collection and specimens stored at  $-70^{\circ}\text{C}$  for 24 and 48 hours (Figure 2D). The viable rates for specimens processed after 24 and 48 hours was 12% and 3.8% respectively (Figure 2D).



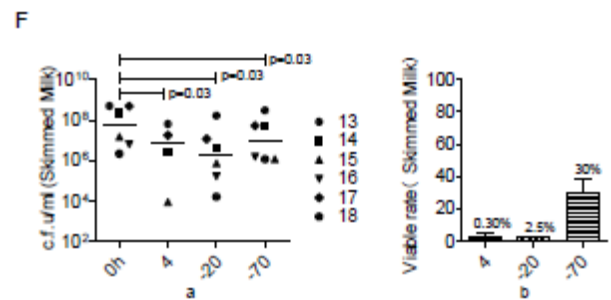
**Figure 2D:** (a), Sputum bacterial density of specimens processed right after collection and after storage 24 and 48 hours at  $-70^{\circ}\text{C}$ , D (b), the viable rates of samples stored in different conditions.

There was a significant reduction in *H. influenzae* density between the specimen processed right after collection and specimens stored neat at  $4^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$  for 7 days (Figure 2E). The viable rates for specimens stored neat at  $4^{\circ}\text{C}$  was 0.36%, at  $-20^{\circ}\text{C}$  was 0.64%, and at  $-70^{\circ}\text{C}$  was 9.11% (Figure 2E).



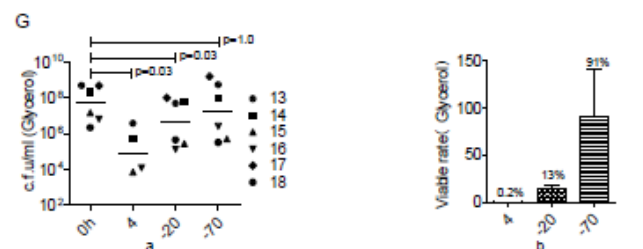
**Figure 2E:** (a) Sputum bacterial density of samples stored neat for 7 days, E (b), the viable rates of samples stored in different conditions.

There was a significant reduction in *H. influenzae* density between the specimen processed right after collection and specimens stored in skimmed milk at  $4^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$  for 7 days (Figure 2F). The viable rates for specimens stored in skimmed milk at  $4^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$  was 3.0%, 2.5% and 30% respectively (Figure 2F).



**Figure 2F:** (a), Sputum bacterial density of 6 samples stored with skimmed milk for 7 days, F (b), the viable rates of samples stored in different conditions.

There was a significant reduction in *H. influenzae* density between the specimen processed right after collection and specimens stored in glycerol at  $4^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$  for 7 days (Figure 2G). There was preserved bacterial density at  $-70^{\circ}\text{C}$  for 7 days. The viable rates for specimens stored in glycerol at  $4^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$  was 0.21%, 13.9% and 90.6% (Figure 2G).



**Figure 2G:** (a) Sputum bacterial density of 6 samples stored with glycerol for 7 days, G (b), the viable rates of samples stored in different conditions.

## Discussion

In practice, due to the time spent on sputum collection and transportation, sample processing time is often delayed in the hospital and laboratory setting. Further studies incorporating a patient empowerment model considering the patient as the key member of the health team and care managers as key health care collaborators may enhance and support services to patients provided by physicians in the primary health care system and facilitate sputum processing [7].

The results showed that there was no significant difference in the density of *H. influenzae* cultured from the samples stored for any time points at room temperature and  $4^{\circ}\text{C}$  following collection. There was however improved viability with samples stored at room temperature. These results indicate that the

preferred storage temperature for sputum samples with *H. influenzae* is room temperature for up to 48 hours. For long term storage of up to 7 days, the sputum sample stored in glycerol at  $-70^{\circ}\text{C}$  led to no significant difference in the density and viability of *H. influenzae*, indicating glycerol as the preferred storage medium at  $-70^{\circ}\text{C}$ .

At room temperature, data on the average viable rates at 24 h and 48 h (110 % and 81 % respectively) were higher than at  $4^{\circ}\text{C}$  (63 % and 46 % respectively), which suggests that room temperature is a better temperature for the storage of sputum sample than  $4^{\circ}\text{C}$ . The study of Murray et al. [7] investigated storage time of sputum samples in bronchiectasis patients chronically infected with *Pseudomonas aeruginosa*. In their study, however, storage at room temperature for 24 and 48 h was associated with a significant increase in bacterial load [8] and they found the optimum storage temperature to be  $4^{\circ}\text{C}$ . Comparing the results of Murray et al. [7] to this study it is clear that the characteristic of various pathogens differ. It is thus important to find the optimum storage conditions to preserve multiple bacterial species from sputum samples.

In the short term storage at  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$ , there was a significant decrease in the density of *H. influenzae* processed after 24 and 48 hours when compared to samples processed right after collection. Haines et al. [8] studied the effect of freezing on several kinds of bacteria. They found that 82% of *Pseudomonas aeruginosa* and 60% of *Achromobacter* were killed by a single freezing and thawing cycle [8]. Our results showed, that 92-96% of *H. influenzae* was killed after storage at  $-20^{\circ}\text{C}$  for 24 or 48 hours and nearly 88-96 % were killed at  $-70^{\circ}\text{C}$ , which shows that the major harm to *H. influenzae* is caused by freezing and thawing. The storage medium, however, can influence *H. influenzae* viability during freezing and thawing.

In our long term storage experiments, two mediums were compared to neat freezing: glycerol and skimmed milk. The results show, that after 7 days of storage before processing, only the sample stored at  $-70^{\circ}\text{C}$  in glycerol showed no significant difference in the density of *H. influenzae* and a viable rate of 91%. All the other storage conditions (Sputum neat at  $4^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ ,  $-70^{\circ}\text{C}$ ; Sputum in skimmed milk at  $4^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ ,  $-70^{\circ}\text{C}$ ; Sputum in glycerol at  $4^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ ) showed a significant reduction in the density of *H. influenzae* and viable rate. Glycerol shows a protective role at increasing the consistency of the sputum and its freezing point, which prevents the formation of ice crystals. In addition independent of the storage medium used, the viable rate was higher at  $-70^{\circ}\text{C}$ , which suggests that the lower temperature is better for long term sputum storage. Very low

temperatures, like liquid nitrogen ( $-196^{\circ}\text{C}$ ) may be the best choice for sputum storage, but further studies are needed [9].

A limitation of the study was the small sample size. The patients however were well characterized and excluded confounders, in particular those on long term antibiotics, as this could have skewed the results. We have also not explored long term storage more than seven days and further studies are needed.

In conclusion, this study shows that for short term storage of sputum from bronchiectasis patients chronically infected with *H. influenzae*, the ideal sputum storage condition is room temperature up to 48h. For long term storage, the optimal condition is storage in glycerol at  $-70^{\circ}\text{C}$ .

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