Do processing time and storage of sputum influence quantitative bacteriology in bronchiectasis?

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This study aimed to establish whether the bacterial density of spontaneous sputum is affected by the time and mode of sample storage. Ten patients with bronchiectasis collected all sputum expectorated over 45 min. The samples were aliquoted and processed at 25 °C for qualitative and quantitative bacteriology at 1, 2, 4 and 6 h from expectoration. Further aliquots were stored at 25 °C, 4 °C and –20 °C for 24 and 48 h prior to processing. The species present was identified and median (interquartile range) sputum log₁₀ bacterial density (c.f.u. ml^{–1}) calculated. All samples cultured grew *Pseudomonas aeruginosa* and for two patients *Staphylococcus aureus* additionally grew for all samples. There was no significant difference in *P. aeruginosa* density in samples processed at 1, 2, 4 and 6 h following expectoration [8.2 (7.8–8.3) c.f.u. ml^{–1}, 8.0 (7.8–8.3) c.f.u. ml^{–1}, 8.0 (7.9–8.2) c.f.u. ml^{–1}, 8.1 (7.9–8.2) c.f.u. ml^{–1}, respectively, *P*=0.392]. Storage for 24 and 48 h at 4 °C did not significantly change the bacterial load compared with processing at 1 h [8.03 (7.6–8.2) c.f.u. ml^{–1}, *P*=0.07, and 7.96 (7.49–8.22) c.f.u. ml^{–1}, *P*=0.09, respectively]. Storage for 24 and 48 h at –20 °C significantly reduced *P. aeruginosa* density [7.1 (6.1–7.7) c.f.u. ml^{–1}, *P*=0.005, and 6.9 (6.2–7.6) c.f.u. ml^{–1}, *P*=0.008, respectively]. Storage at 25 °C for 24 and 48 h was associated with a significant increase in bacterial load [8.3 (8.1–8.6) c.f.u. ml^{–1}, *P*=0.009, and 8.4 (8.1–8.5) c.f.u. ml^{–1}, *P*=0.03, respectively]. Bacterial density was not affected by storage for up to 6 h following expectoration at 25 °C; beyond this, storage at 4 °C is preferred.

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INTRODUCTION

Sputum microbiology has a crucial role in both the diagnosis and management of non-cystic fibrosis bronchiectasis (Angrill *et al.*, 2002; Loebinger *et al.*, 2009; Murray & Hill, 2009). During routine care of patients with pulmonary exacerbations and during multicentre trials of new antimicrobial agents, quantitative bacteriology of sputum samples is used to monitor treatment response and assess the efficacy of therapies (Barker *et al.*, 2000; Drobnic *et al.*, 2005). In the UK, this technique is typically limited to reference laboratories, which often implies a prolonged time period from expectoration to processing as samples are transported to such centres. Appropriate collection, storage and transportation of sputum samples is critical as it may have a direct impact on the recovery of viable organisms. Surprisingly, there appear to have been few studies to investigate the impact of sputum storage on bacterial recovery. The aim of this study was to establish

whether the total bacterial density recovered and the bacterial species identified from spontaneous sputum is affected by the mode of storage and time of processing of up to 48 h from expectoration.

METHODS

This was a prospective study of patients with clinically stable non-cystic fibrosis bronchiectasis that were chronically colonized with *Pseudomonas aeruginosa* (May–June 2009). The study was approved by the Lothian Research Ethics Committee. The primary outcome was sputum *P. aeruginosa* density (log₁₀ c.f.u. ml^{–1}).

Patients.

Patients were recruited from the Bronchiectasis Clinic, Royal Infirmary of Edinburgh, Scotland, UK. Inclusion criteria were: a radiological diagnosis of bronchiectasis by high resolution computed tomography (HRCT) chest scan according to the features described by Naidich *et al.* (1982), chronic sputum expectoration, chronically infected with *P. aeruginosa* (defined as culture of *P. aeruginosa* from sputum on at least three consecutive occasions in the preceding 12 months when clinically stable), clinically stable disease (defined as no

Abbreviation: HRCT, high resolution computed tomography.

requirement for antibiotics in the 4 weeks preceding study entry). Exclusion criteria were: current or ex-smokers of ≤ 2 years or ex-smokers with a history of ≥ 10 pack years of smoking and emphysema shown on HRCT; on long-term oral or inhaled antibiotics.

Sputum collection.

Patients were instructed to perform cycles of two to three forced expiratory techniques (huffs) followed by coughing to enhance expectoration over a 45 min period. All sputum expectorated was collected in a sterile transparent container.

Sputum storage.

Each sputum sample was immediately aliquoted into 10 separate sterile containers. Qualitative and quantitative bacteriology was then performed at the following time points: 1, 2, 4 and 6 h from the start of expectoration (Fig. 1). The samples were stored at room temperature (25 °C) throughout these time points. The other aliquoted samples were handled as follows: two samples were immediately stored at 4 °C for 24 and 48 h; two samples were immediately stored at -20 °C for 24 and 48 h; two samples were stored at room temperature (25 °C) for 24 and 48 h (Fig. 1).

Sputum processing.

Each sample was confirmed to be a valid sputum sample suitable for processing if there were >25 polymorphonuclear leukocytes and <10 squamous cells present on Gram stain on low power ($\times 100$) magnification (Gleckman *et al.*, 1988). At the designated time for processing, samples were prepared for qualitative and quantitative bacteriology as described by Pye *et al.* (1995). Briefly, sputum was homogenized and liquified using an equal volume of 0.1 % DTT, and serially diluted using sterile 0.9 % saline to achieve dilution factors of 10^{-1} to 10^{-4} . Cetrimide (Difco) *Pseudomonas* isolation agar and blood agar plates were inoculated with 100 μ l sample. These were incubated at 37 °C for 24 h. Colonies were then counted to determine the sputum *P. aeruginosa* density expressed as $\log_{10}$ c.f.u. ml^{-1} . Any other microbial pathogens present were identified and counted. For consistency, all samples were handled, processed and counted by the first author.

Statistics.

Statistical analysis was performed using SPSS for Windows, version 17 (SPSS) and GraphPad Prism version 5.0 (GraphPad Software). Data

are presented as median (interquartile range) values. The Bland–Altman technique was used to compare the different storage methods tested. If a difference was identified, the Wilcoxon analysis for two groups was used. A two-tailed *P* value of <0.05 was considered statistically significant.

RESULTS

Patients

Ten patients (two male) aged 70 (67.2–70.2) years with non-cystic fibrosis bronchiectasis and a history of chronic *P. aeruginosa* colonization were recruited. The aetiology of the bronchiectasis was post-infective in five (50 %), idiopathic in four (40 %) and inactive allergic bronchopulmonary aspergillosis in one (10 %). Five (3.75–5.25) lobes were involved on HRCT chest scan (for the purposes of this study the lingula was considered a separate lobe) and eight (80 %) patients had either cystic or varicose dilatation in at least one lobe.

Baseline qualitative sputum bacteriology

All samples cultured *P. aeruginosa*, of which eight had mucoid species. Two patients additionally had a growth of *Staphylococcus aureus* identified at baseline and this remained present in all subsequent aliquoted sputum samples. No other organisms were present on culture at any time point for the remaining eight patient samples. The patients with mucoid species had advanced bronchiectasis with five (3.25–5.75) lobes involved, and all had either cystic or varicose dilatation in at least one lobe.

Quantitative sputum bacteriology

Sputum processed at 1–6 h from expectoration. There was no significant difference ($P=0.392$) in *P. aeruginosa* density when processing the sputum at 1, 2, 4 and 6 h from expectoration (Fig. 2). The mean difference (sd) with 95 %

Fig. 1. Method of sputum sample storage and processing.

Fig. 2. Bland–Altman plot of sputum bacterial density over (a) 2, (b) 4 and (c) 6 h. The solid lines represent the mean difference between the density at 1, and 2, 4 and 6 h for (a), (b) and (c), respectively. The perforated lines represent the upper and lower 95 % limits of agreement.

limits of agreement between processing at 1 h compared with 2 h was -0.04 (0.31), -0.65 to 0.57 ; between 1 and 4 h was -0.1 (0.54), -1.2 to 0.97 and between 1 and 6 h was -0.06 (0.35), -0.7 to 0.6 .

Sputum processed after storage for 24 h. When comparing sputum processed at 1 h from the start of expectoration with sputum stored for 24 h at room temperature (25°C), there was a small but significant increase in *P. aeruginosa* density at 24 h (Fig. 3). There was no significant difference in *P. aeruginosa* density between sputum processed at 1 h with sputum stored for 24 h at 4°C prior to processing. When comparing sputum processed at 1 h with sputum processed after storage at -20°C for 24 h, there was a significant reduction in *Pseudomonas* density. The mean difference (SD) with 95 % limits of agreement between processing at 1 h compared with 24 h storage at room temperature (25°C) was 0.38 (0.39), -0.39 to 1.16 ; between 1 and 24 h at 4°C was

Fig. 3. Bland–Altman plot of sputum bacterial density after 24 h storage at (a) 25°C , (b) 4°C and (c) -20°C . The solid lines represent the mean difference between the density at 1 h and after storage for 24 h at 25°C , 4°C and -20°C for (a), (b) and (c), respectively. The perforated lines represent the upper and lower 95 % limits of agreement.

-0.12 (0.24), -0.6 to 0.36 ; and between 1 and 24 h at -20°C was -1.05 (0.84), -2.7 to 0.60 .

Sputum processed after storage for 48 h. When comparing sputum processed at 1 h from the start of expectoration with sputum stored for 48 h at room temperature (25°C), there was a significant increase in *P. aeruginosa* density at 48 h (Fig. 4). There was no significant difference between sputum processed at 1 h with sputum stored for 48 h at 4°C prior to processing. When comparing sputum processed at 1 h with sputum processed after storage at -20°C for 48 h, there was a significant reduction in *P. aeruginosa* density. The mean difference (SD) with 95 % limits of agreement between processing at 1 h compared with 48 h storage at room temperature (25°C) was 0.37 (0.51), -0.64 to 1.38 ; between 1 and 48 h at 4°C was -0.16 (0.28), -0.72 to 0.40 ; and between 1 and 48 h at -20°C was -1.07 (0.92), -2.88 to 0.74 .

Mucoid *P. aeruginosa* samples. Samples culturing only mucoid *P. aeruginosa* were analysed ($n=8$). The median

Fig. 4. Bland–Altman plot of sputum bacterial density after 48 h storage at (a) 25, (b) 4 and (c) -20°C . The solid lines represent the mean difference between the density at 1 h and after storage for 48 h at 25, 4 and -20°C for (a), (b) and (c), respectively. The perforated lines represent the upper and lower 95% limits of agreement.

(interquartile range) sputum $\log_{10}$ bacterial density of samples at 1 h was 8.25 (7.94–8.84) c.f.u. ml^{-1} . After storage for 24 h at room temperature (25°C) the density was slightly higher 8.47 (8.12–8.65) c.f.u. ml^{-1} ($P=0.01$); after 24 h storage at 4°C the density was not significantly different 8.04 (7.77–8.19) c.f.u. ml^{-1} ($P=0.16$) and after 24 h storage at -20°C the density was lower 7.38 (6.16–7.78) c.f.u. ml^{-1} ($P=0.01$). After storage for 48 h at room temperature (25°C), the density was slightly higher 8.44 (8.1–8.49) c.f.u. ml^{-1} ($P=0.02$); after 48 h storage at 4°C the density was not significantly different 8.06 (7.7–8.25) c.f.u. ml^{-1} ($P=0.26$) and after 48 h storage at -20°C , the density was lower 7.3 (6.40–7.70) c.f.u. ml^{-1} ($P=0.02$).

DISCUSSION

This study showed that *P. aeruginosa* density in non-cystic fibrosis bronchiectasis is not affected if sputum is processed within 6 h following expectoration when stored at room temperature (25°C). The preferred environment for

samples requiring storage for either 24 or 48 h with no significant change in bacterial density or identity compared with processing within 1 h is at 4°C . However, storage of sputum samples for 24 and 48 h at -20°C significantly reduces *P. aeruginosa* density, while storage at room temperature (25°C) causes a small but statistically significant increase in density but no change in species identified. Subanalysis of patients with mucoid *P. aeruginosa* alone did not change these findings.

It is widely accepted that sputum samples should be processed as quickly as possible following expectoration to optimize pathogen recovery. This may often be logistically difficult, particularly when quantitative bacteriology is necessary and the sample needs transported to a tertiary centre. This study provides useful evidence for both mode of storage during transportation and appropriate time for processing.

We selected patients known to be colonized with *P. aeruginosa* as it is a common pathogen in non-cystic fibrosis bronchiectasis, and it is associated with more severe disease and as such is frequently the target of antimicrobial agents (Evans *et al.*, 1996; Martinez-Garcia *et al.*, 2007; Wilson *et al.*, 1997). We found a small but statistically significant rise in bacterial density when the sputum was stored at room temperature for 24 and 48 h. In their study of sputum storage, Pye and colleagues did not observe any difference between viable numbers of pathogens retrieved following 24 h storage at 20°C compared with samples processed on the same day of expectoration, [mean (SEM) 1.21×10^9 (0.35) c.f.u. ml^{-1} for same day samples (exact time of processing from expectoration not specified) versus 1.15×10^9 (0.5) c.f.u. ml^{-1} for samples stored at 20°C for 24 h] but their study included multiple pathogens and was not specific for *P. aeruginosa* (Pye *et al.*, 2008).

Our findings for storage at 4°C are similar to the study of Wong *et al.* (1984) of cystic fibrosis paediatric sputum samples, which found no detectable change in the quantitative bacterial load of several different pathogens, including *P. aeruginosa*, after 2 days storage of homogenized sputum at 4°C . Additionally, in their study Gould *et al.* (1996) processed sputum samples on the day of receipt in the laboratory, and subsequently stored the processed samples for 48 h at 4°C for repeat qualitative bacteriology and achieved reproducible results in up to 75% of samples, although the patient population for the samples was not described and quantitative bacteriology was not performed.

These previous studies provided useful information and prompted us to investigate further. Our study is believed to be unique in exploring the effect of sample processing, at varying times on the same day of expectoration as well as up to 48 h with storage of the raw sample at three different temperatures, on sputum *P. aeruginosa* density in patients with non-cystic fibrosis bronchiectasis. Our study is particularly tailored to the logistics of clinical practice and the development of laboratory guidelines. In both

primary and secondary care, there is often a delay in transportation of samples to the local laboratory – a previous study found that within an individual hospital the mean time from collection to processing was 2 h, with a range from several minutes to 13 h (Jacobson *et al.*, 1981). Our findings can reassure the clinician responsible for sample collection that given correct storage conditions, a sputum sample may remain viable and reliably interpreted for its results for *Pseudomonas* infection up to 48 h from expectoration if stored at 4 °C. Furthermore, multicentre studies in both cystic fibrosis and non-cystic fibrosis bronchiectasis often utilize quantitative bacterial load as an end point, thus optimum storage and processing of samples for quantitative culture is important (Bilton *et al.*, 2006; Hodson *et al.*, 2002). However, further studies are needed to explore the effect of sample storage and processing time on other pathogenic organisms.

In conclusion, sputum samples from patients with non-cystic fibrosis bronchiectasis stored at room temperature and processed up to 6 h from expectoration can be reliably interpreted for *P. aeruginosa* density. For samples requiring processing beyond this time, the optimum environment for storage is at 4 °C for up to 48 h. Freezing of sputum at –20 °C for 24 and 48 h has a significantly negative impact on culture of *P. aeruginosa*.

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