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Homogenisation of cystic fibrosis sputum by sonication — An essential step for *Aspergillus* PCR

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ABSTRACT

The importance of *Aspergillus* as a lung pathogen in cystic fibrosis (CF) is becoming increasingly recognised. However, fungal culture of CF sputum is unreliable and there is no consensus for identifying phenotypes beyond ABPA that may benefit from antifungal therapy. There are no published studies using real-time PCR to detect *Aspergillus* in CF sputum. The major barrier to sensitive detection of *Aspergillus* using PCR is sputum homogenisation. This study aimed to optimise sputum homogenisation utilising sonication to improve *Aspergillus* DNA extraction. Sonication amplitude and duration that enabled sputum homogenisation but ensured preservation of DNA integrity were first determined. 160 sputum samples were collected from CF patients. 49 of the sputum samples were split, one half was used for standard culture and the other half was homogenised with NALC–NaOH before undergoing DNA extraction. The subsequent 111 samples were homogenised with dithiothreitol plus sonication prior to culture and DNA extraction. Real-time PCR targeting a portion of the 18S rDNA of *Aspergillus* was performed on all DNA extractions. In the 49 samples with no sonication 8 (16%) were culture positive but only 4 of these were PCR positive. However, PCR was positive in 11 culture negative samples. PCR after sonication showed a significant improvement in sensitivity: 33 (30%) were culture and PCR positive, 48 (43%) were culture negative, but PCR positive ($p<0.0001$) and 30 (27%) were culture and PCR negative. The combination of dithiothreitol and sonication to homogenise sputum increases PCR yield, with PCR being substantially more sensitive than culture.

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1. Introduction

Cystic fibrosis (CF) is a common genetic condition which primarily affects water and ion transport across epithelial surfaces. The major cause of morbidity and mortality is pulmonary disease. Pulmonary secretions are thick and respiratory cilia have impaired movement. Recurrent bacterial infections cause progressive damage to the lungs. Although bacterial infections are responsible for the majority of this pulmonary damage there is an increasing recognition of the role of fungal infections. The most common fungus identified in the secretions of CF patients is *Aspergillus fumigatus* (Bakare et al., 2003).

Aspergilllus is a ubiquitous fungus that causes a number of different clinical presentations in CF. These include allergic bronchopulmonary aspergillosis (ABPA), allergic sensitisation, aspergilloma and invasive aspergillosis (IA) (Stevens et al., 2003). Aspergillus bronchitis in CF has been described more recently (Shoseyov et al., 2006) but criteria for diagnosis and treatment are unclear. Allergic hypersensitivity is the most common presentation in the immunocompetent host. Although there is growing evidence for the use of antifungals in ABPA (Nepomuceno et al., 1999; Skov et al., 2002; Stevens et al., 2000), there have been no studies to evaluate if there is benefit from antifungal treatment for patients colonised with or sensitised to *Aspergillus*. Colonisation is linked to risk of hospitalisation and lower lung function (Amin et al., 2010) but causality is in question and prospective data is required. Sensitisation has been linked to lower lung function in CF (Kraemer et al., 2006) and non-CF asthmatic patients (Fairs et al., 2010). In order to establish if *Aspergillus* colonisation and sensitisation have a pathogenic role in lung function decline, and to monitor antifungal treatment, accurate methods to detect *Aspergillus* in CF respiratory secretions are needed.

CF sputum is often extremely viscous and difficult to liquefy. The optimal method for culture is unknown and varies as different organisms thrive in different growth conditions (Borman et al., 2010). Techniques such as selection of purulent material, bedside gram stain and culture, selective media, homogenisation and quantitative cultures have been attempted to optimise culture yields. Organisms growing in biofilms are also difficult to grow (Randall, 2008). Molecular methods of identification have shown that culture only

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identifies a minority of organisms present (Harris et al., 2007; Rogers et al., 2004). These factors account for the wide variation in reported prevalence of *Aspergillus* in CF sputum (5.6–57%) (Bakare et al., 2003).

Real-time PCR has been used in the identification of *Aspergillus* in bronchoalveolar lavage (BAL) and blood samples to aid the rapid diagnosis of IA (Mengoli et al., 2009; Tuon, 2007). However, real-time PCR has not been used for the detection of *Aspergillus* in CF sputum. PCR depends greatly on successful DNA extraction. There is no published literature on optimal *Aspergillus* DNA extraction from CF sputum. In CF, the most notable barriers to DNA extraction are sputum homogenisation, the presence of biofilms and the presence of inhibiting substances such as inhaled antibiotics. *Aspergillus* also has a much tougher cell wall than bacteria which must be broken to expose DNA. Sputum homogenisation to free and concentrate *Aspergillus* from the large, complex volume of mucin and biofilm matrix in CF sputum is essential prior to DNA extraction. Chemical homogenisation does not always penetrate this matrix adequately so, if PCR is to be successful, mechanical homogenisation, which does not destroy DNA integrity, is needed. Sonication is an inexpensive and rapid method to homogenise clinical specimens. It has been used with success to improve the culture sensitivity of *Mycobacterium* spp. and anaerobes from sputum (Jewes and Spencer, 1990; Sparham et al., 1978). This study aimed to develop an optimal method to homogenise CF sputum, comparing sonication with chemical homogenisation, prior to *Aspergillus* DNA extraction and PCR.

2. Materials and methods

2.1. Ethics

Approval for this study was obtained from the Manchester local research ethics committee (REC ref 07/Q1403/70).

2.2. Patient sample collection

160 adult CF patients gave informed written consent to participate in the study. A sputum sample, of between 1 and 10 mL, was collected from each patient when they attended the Manchester Adult CF Unit (MACFU) outpatient department. Patients expectorated sputum samples spontaneously with no sputum induction or bronchoscopy.

2.3. DNA extraction and real time PCR

DNA extraction was performed for all experiments, according to the manufacturer's protocol, using the MycXtra™ DNA extraction kit (Myconostica, Manchester, UK). This kit uses chemical homogenisation of sputum followed by centrifugal concentration of fungal spores and hyphae. DNA is released by bead beating and, after removal of inhibiting substances, is bound to a silica filter which is washed to suspend DNA in 40 μL of water. *Aspergillus* DNA was detected in all experiments using the real-time molecular beacon PCR assay, MycAssay™ Aspergillus (Myconostica, Manchester, UK) on a Smartcycler® system (Cepheid, Sunnyvale, CA, USA). The target primer sequence for this kit is a portion of the ribosomal 18S gene. Manufacturer's instructions were followed with the exception that after amplification, the stated cut-off value was disregarded and Ct values interrogated. *A. fumigatus* and at least 14 different pathogenic *Aspergillus* spp. are detected with this assay, which may also detect *Penicillium* spp. The kit contains positive and negative controls as well as an internal amplification control sequence to indicate if PCR inhibitors are present in the sample. The assay limit of blank is a crossing threshold (Ct) of 38 cycles with a target sensitivity of <50 18S copies, approximately 1 genome, as from 37 to 90 18S copies are present in different isolates of *A. fumigatus* (Herrera et al., 2009).

2.4. Culture and DNA extraction protocol 1

The first 49 patient sputum samples underwent culture and extraction protocol 1. Each fresh sputum sample was split approximately in half according to volume. An equal volume of Sputasol (dithiothreitol 1.4%) (Oxoid Ltd., Basingstoke, UK) was added to one half and sputum culture was performed according to the National Standards Method BSOP 57 produced by the Health Protection Agency (www.hpa-standardmethods.org/documents/bsop/ pdf/bsop57.pdf) but modified to plate 10 μL rather than 1 μL of each sample onto each of 3 sabouraud dextrose with chloramphenicol agar plates (SABC) (Oxoid, Bassingstoke, UK) agars and incubated at 25, 37 and 45 °C for 48 h. Fungal colonies were counted and identified by microscopy using lactophenol cotton blue. After culture, this half of the sputum sample was discarded. The remaining half underwent fungal DNA extraction. The MycXtra™ DNA extraction kit recommends the use of BBL™ Mycoprep™ (Becton, Dickinson and Company, NJ, USA) to homogenise sputum prior to DNA extraction. The principle liquefying agents in this preparation are N-Acetyl-Cysteine and sodium hydroxide (NALC/NaOH). Twice volume BBL™ Mycoprep™ was added to the second half of the sputum sample and neutralised with phosphate buffer as per manufacturer's instructions. The sample was then heated to 80 °C for 20 min to ensure mycobacterial decontamination. Once cooled, fungal DNA was extracted and detected using real-time PCR as described above.

2.5. Chemical homogenisation comparison

The ability of NALC/NaOH to homogenise sputum for DNA extraction and PCR was compared to dithiothreitol. The use of dithiothreitol alone prevents sample splitting while allowing comparative culture to PCR. In order to represent CF sputum consistency, sputum was collected from a CF patient known to have never grown *Aspergillus*. This fresh sample was split into 4 equal volumes using a pipette. Two of these samples were spiked with 1 mL of sterile DNA free water containing 0.2 ng of *A. fumigatus* (AF293) DNA, and the other two were spiked with 1 mL of sterile DNA free water containing 1×10^5 AF293 spores, counted using a haemocytometer. One DNA spiked and one spore spiked sample were liquefied by adding an equal volume of Sputasol then agitating and incubating as per manufacturer's protocol. The remaining two samples were liquefied with an equal volume of BBL™ Mycoprep™, left to stand at room temperature for 15 min then neutralised with phosphate buffer according to the manufacturer's protocol. Fungal DNA was then extracted and detected in all samples using real-time PCR.

2.6. Spore sonication

Five mL of sterile water was added to a 50 mL centrifuge tube and spiked with 1×10^5 AF293 spores. The tube was placed on ice in a polystyrene box to prevent the heat produced by sonication melting the plastic centrifuge tube. A glass container should not be used as it can be broken by sonication. Sonication was performed using a Sonics® VC505 ultrasonic processor (Sonics and Materials Inc., Newtown, CT, USA) in a sound abating enclosure. Sonication produces a high pitched noise due to harmonics emanating from the fluid and container walls. An enclosure reduces this noise and protects the operator from accidental spillage. For this ultrasonic processor, a 3 mm microtip is recommended to process samples with volumes from 250 μL to 10 mL. A four element coupler with 3 mm stepped microtips was used to allow 4 samples to be processed simultaneously. The maximum output amplitude (peak to peak displacement) that can be applied to this type of microtip is 40% which, when used in a 4 element coupler, produces an amplitude of 295 μm. The amplitude is a measure of the intensity of the sonication. By reducing the output amplitude to below 40% the intensity will be lower and time to homogenisation longer. Therefore, the maximum output

was initially selected in order to keep sonication times to a minimum. The effect of sonication duration, at this amplitude, on spore viability and cell wall integrity was assessed by sonicating the same spore sample at 30 second intervals up to 120 s. After each 30 second sonication interval, 10 μL was examined under microscopy. Additionally after each 30 second sonication interval a 10 μL loop of the sample was inoculated onto SABC agar and incubated at 37 °C for 48 h. The number of colony forming units (CFU) was noted during this incubation period.

2.7. Hyphae sonication

One hundred mL of sabouraud dextrose broth (Oxoid) was added to a 250 mL glass conical flask, spiked with 1×10^5 AF293 spores and capped with a rubber bung. The flask was placed in a shaker incubator (311 DS, Appleton Woods, UK) at a speed of 170 rpm and temperature of 37 °C for 48 h. After 48 h 1 hyphal ball, measuring approximately 5 mm in diameter, was removed and added to a 50 mL centrifuge tube containing 5 mL of sterile water. This was sonicated as described above for spore sonication and microscopy slides plus SABC agar plates were made after each sonication interval.

2.8. Sputum sonication

Sputum was collected from 2 CF patients chronically colonised with *A. fumigatus*. An equal volume of Sputasol was added to each sample and incubated for 30 min at 37 °C, with periodic shaking. A 10 μL loop of the sample was then inoculated onto SABC agar and incubated at 37 °C for 48 h. After culture, each sample was then transferred to a 50 mL centrifuge tube and placed on ice. Sonication, with culture after each interval, was then performed as described for spore sonication.

2.9. Chemical homogenisation with additional sonication

Sputum was collected from 4 adult CF patients known to be chronically colonised with *A. fumigatus*. Each sample was split in half according to volume collected. An equal volume of Sputasol was added to each half. One half of the sample was sonicated until fully homogenised while the other half had no further homogenisation. Fungal DNA was then extracted and detected in all samples using real-time PCR.

2.10. Culture and DNA extraction protocol 2

The subsequent 111 patient samples collected were processed using culture and DNA extraction protocol 2. Each whole fresh sample was liquefied with Sputasol and culture was performed as for protocol 1. After culture, each whole sample was then sonicated for 120 s at an amplitude of 295 μm. Sonication was followed by further culture of 10 μL of the sample. The entire remaining sample was heated to 80 °C for 20 min then fungal DNA was extracted and detected using real-time PCR.

3. Results

3.1. Culture and DNA extraction protocol 1

In the first 49 patient samples, undergoing chemical homogenisation alone, 8 were culture positive for *A. fumigatus* and 13 were PCR positive for *Aspergillus* spp. However, only 4 of the 8 culture positive samples were PCR positive (Table 1). This was not significant, $p=0.227$ (Fisher's exact).

Table 1
Chemical homogenisation followed by *Aspergillus* culture and PCR ($n=49$).

	PCR positive	PCR negative
Culture positive	4	4
Culture negative	11	30

3.2. Chemical homogenisation comparison

Neither dithiothreitol nor NALC/NaOH led to the complete homogenisation of sputum. However, the use of dithiothreitol treatment improved Ct values for samples spiked both with AF293 DNA and AF293 spores compared to NALC/NaOH treatment. Sputum spiked with spores: dithiothreitol PCR Ct 28.4 and NALC/NaOH PCR Ct 31.8. Sputum spiked with DNA: dithiothreitol PCR Ct 32.4 and NALC/NaOH PCR Ct 33.8.

3.3. Spore sonication

There was no evidence of spore fragmentation on microscopy after 120 s of sonication at an amplitude of 295 μm. There was no inhibition of spore growth after 120 s of sonication (Fig. 1). There was therefore no evidence of cell wall or DNA fragmentation using this protocol of sonication up to 120 s.

3.4. Hyphae sonication

Microscopy demonstrated increasing hyphal breakdown with increasing sonication time. This was reflected by culture which also demonstrated an increasing number of CFUs (Fig. 2). The normal colony morphology and increasing CFU count provided evidence that there was no DNA destruction up to 120 s of sonication.

3.5. Sputum sonication

Both CF sputum samples were fully homogenised after the addition of dithiothreitol and 120 s of sonication. There was no inhibition of growth of *A. fumigatus* from CF sputum after sonication. There was an increase in CFUs with culture after sonication (Fig. 3).

3.6. Chemical homogenisation with additional sonication

Sputum was fully homogenised in all samples after 120 s but not always before this.

Addition of sonication to dithiothreitol homogenisation improved *Aspergillus* PCR Ct value in 3 samples, with a mean reduction in Ct value of 4.25 cycles, and allowed detection of *Aspergillus* DNA in 1 sample that was PCR negative prior to sonication ($p=0.06$, Wilcoxon signed rank test, $Z=1.826$) (Table 2).

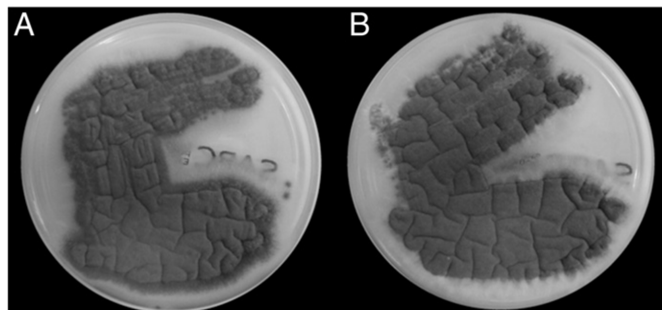

Fig. 1. AF293 spore growth after (A) 0 and (B) 120 s of sonication.

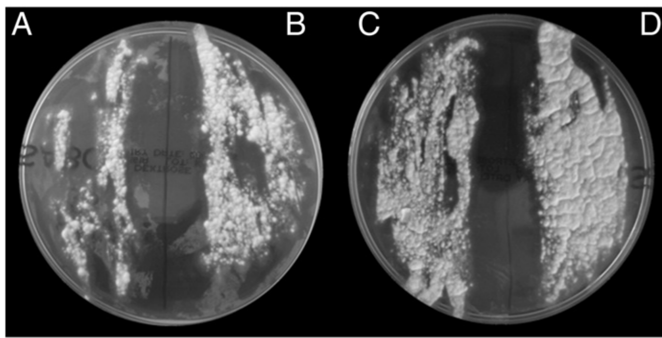

Fig. 2. AF293 hyphae growth after (A) 30, (B) 60, (C) 90 and (D) 120 s sonication.

3.7. Culture and DNA extraction protocol 2

In the subsequent 111 patient sputum samples, undergoing homogenisation with dithiothreitol plus sonication, 21 (19%) were culture positive for *A. fumigatus* by standard culture. One sample additionally grew *Penicillium* species and 2 grew *Penicillium* species alone. After sonication 33 (30%) of the 111 samples grew *A. fumigatus*, $p = 0.042$ (Chi-square). 1 sample additionally grew *A. flavus*, and 1 additionally grew *Penicillium* species. PCR was positive in 81 of the 111 samples (73%). All *A. fumigatus* culture positive samples were PCR positive. The 2 samples growing *Penicillium* species alone were PCR negative. 48 culture negative samples were PCR positive (Table 3) ($p<0.0001$ (Fisher's exact)). It was noted that sonication after dithiothreitol treatment not only improved *A. fumigatus* detection but also significantly increased CFUs cultured (Table 4) compared with dithiothreitol treatment alone (Wilcoxon signed rank test, $Z = 4.15$, $p = <0.0001$). Of note however, in 4 instances, sputum sonicated after dithiothreitol treatment was culture negative when the same sample treated only with dithiothreitol was culture positive, although PCR was strongly positive in all 4 cases. These are shown as the first 4 samples in Table 4. There was no correlation between PCR Ct value and CFUs identified on culture ($r = -0.07$) (Fig. 4).

4. Discussion

This study has addressed the known barriers to *Aspergillus* DNA extraction from CF sputum, in particular sputum homogenisation, biofilm breakdown and removal of inhibiting substances. CF patients often produce large volumes of thick, dehydrated sputum. Release of *Aspergillus* from this complex matrix must be achieved in an effective and time efficient manner to then allow DNA extraction from a small

Table 2
Effect of adding sonication to dithiothreitol on *Aspergillus* PCR.

Sample ID	Sonication (Y/N)	PCR result	PCR Ct value
CF031-1	N	Positive	34.5
CF031-2	Y	Positive	29.6
CF337-1	N	Positive	25.9
CF337-2	Y	Positive	22.4
CF160-1	N	Positive	33.8
CF160-2	Y	Positive	31.3
CF008-1	N	Negative	>38
CF008-2	Y	Positive	34.1

concentrated volume. The data presented shows that chemical homogenisation alone leads to poor PCR sensitivity but the addition of the mechanical forces of sonication achieves more sensitive PCR results than standard culture. Initial chemical liquification remains essential as CF sputum is often too dehydrated to allow sole use of sonication. Dithiothreitol plus sonication, performed for 120 s at an amplitude of 295 μm, fully homogenises all CF sputum samples but does not have any adverse effect on *Aspergillus* growth or DNA integrity and can significantly improve standard culture yields ($p = <0.0001$). Despite this strong correlation, 4 patient samples were culture negative after sonication but culture positive prior to sonication. These 4 samples appear first in Table 4 and demonstrate that, in each case, the number of CFUs were low (1, 2, 2, and 3), representing less than 300 organisms per mL of sample. Therefore, this discrepancy likely represents and highlights the inaccuracy of culture based techniques which use only a small proportion of the whole sample leading to sampling error. Sonication is able to break down complex hyphal structures and biofilms so it may improve DNA quantification. This study only compared the reduction in Ct values in 4 patient samples but the data did confirm a trend towards reduced Ct values (higher DNA yield) with sonication ($p = 0.06$). Further studies of repeatability and clinical relevance of differing Ct values should precede extensive optimisation of sonication conditions.

A sonication probe converts sound energy to high frequency vibrations at the probe tip. These vibrations create small spaces or 'micro-cavities' in the nearby solution as molecules are forced away from the probe. Eventually they reach a point where the sonication wave no longer keeps them apart and the cavities collapse inwards producing another intense shockwave that spreads with similar effect. The creation of cavities creates a positive pressure while their collapse creates negative pressure. The rapid alternation of pressures travelling through the liquid results in the destructive capabilities of sonication. Sonication can be used to break down cell walls to release DNA for PCR (Hamilton and Sandin, 1999) but this study aimed to determine only the amplitude

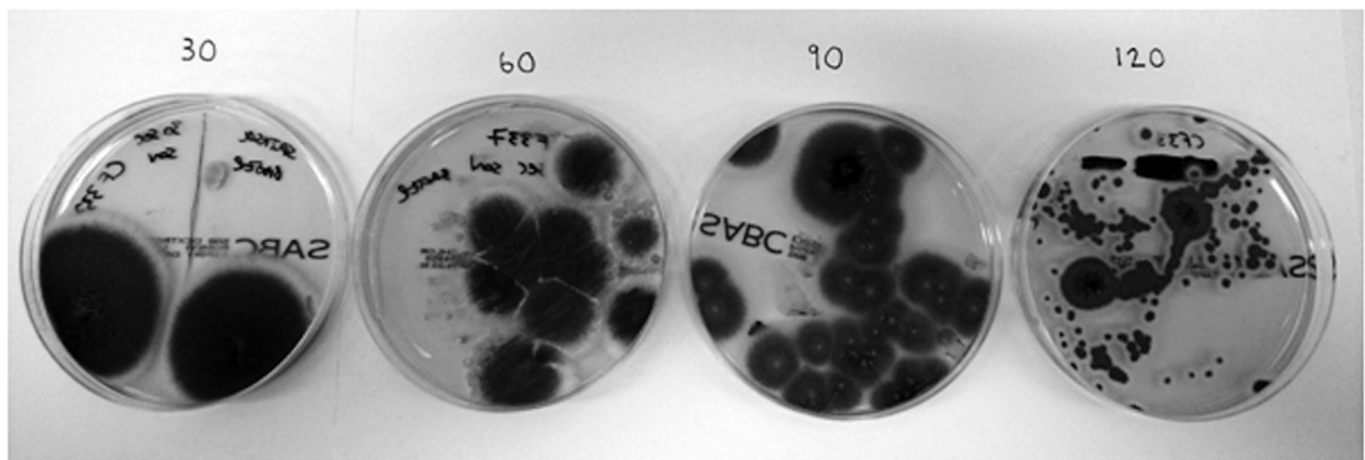

Fig. 3. *A. fumigatus* growth from CF sputum after 30, 60, 90 and 120 s sonication.

Table 3
Dithiothreitol plus sonication homogenisation followed by *Aspergillus* culture and PCR (n=111) (p=<0.0001).

	PCR positive	PCR negative
Culture positive	33	0
Culture negative	48	30

and duration of sonication to break down sputum mucins and biofilms and not the *Aspergillus* cell wall. This was done because excess amplitude or duration of sonication is capable of disrupting DNA integrity once the cell wall is broken. If the cell wall was to be broken to extract but not destroy DNA, exact amplitudes would need to be calculated for each individual patient sample depending on initial sputum consistency. As there are now simple, effective methods of cell wall breakdown in most commercial DNA extraction kits, such as the MycXtra™ kit, this step does not need to be done by sonication. This sonication protocol ensures full homogenisation of sputum but no *Aspergillus* cell wall destruction. This study did not test lower amplitudes of sonication as there was no cell wall disruption or DNA breakdown at the maximum amplitude for the appropriate sample volume microtip. This protocol cannot be recommended for organisms other than *Aspergillus* as the cell walls of different genera may fracture at this sonication amplitude and duration resulting in differing culture yield and possibly PCR signal.

This study has shown that dithiothreitol can be used as the liquefying agent prior to DNA extraction. Independent of sonication, the data suggests that it may improve DNA extraction over NALC/NaOH, which is recommended for use with the MycXtra™ kit. However, further studies are required to confirm this observation. The direct comparison of these two liquefying agents was only done in 2 samples so it lacks statistical power for comparison. Furthermore, the use of a haemocytometer to determine spore counts is subject to an error rate of between 5 and 20% (Hamilton, 1955). This error rate is less for DNA quantification by spectrometer and it was noted that the difference in Ct values was less for the DNA spiked samples than the spore spiked samples. Further samples were not compared as the mechanical forces of sonication were shown to be superior to either dithiothreitol or NALC/NaOH alone. Dithiothreitol may improve DNA extraction over NALC/NaOH due to improved homogenisation as dithiothreitol has been shown to significantly decrease sputum viscosity compared to NALC (Hirsch et al., 1969). Dithiothreitol may also improve DNA extraction, independent of sonication, as it allows the use of smaller sample volumes prior to DNA extraction. The use of NALC/NaOH to homogenise sputum requires large volumes of phosphate buffer to neutralise the sample prior to DNA extraction, up to 50 mL. A neutral pH is essential to prevent PCR inhibition. The first step of DNA extraction is to centrifuge the sample to concentrate cells, and then the supernatant is discarded. Dithiothreitol has a pH of 7.4 and requires no neutralisation so volumes remain small. We cultured the supernatant in 8 samples and found it to still contain fungal material when NALC/NaOH was used but not when dithiothreitol was used. Discarding this material leads to a loss of DNA which may reduce PCR sensitivity. Lastly, dithiothreitol prevents the need for sample splitting to achieve both culture and PCR which is important as standard culture is still desirable to allow fungal sensitivities to be performed. NALC/NaOH has the ability to kill many organisms including *Aspergillus* but dithiothreitol has been shown not to affect bacterial or fungal counts (Pye et al., 1995) leading to its routine use in many laboratories. Sample splitting is also undesirable as chemical homogenisation alone does not lead to an even distribution of fungal material. It is not yet known if the addition of sonication can overcome this and allow sample splitting. This is important to establish as a standard volume of sputum must be used for PCR if Ct values are to be compared and interpreted in the clinical setting.

Table 4
CFUs with dithiothreitol alone and dithiothreitol plus sonication, compared with real-time PCR signal (expressed as Ct value).

Patient ID	CFU dithiothreitol only	CFU dithiothreitol plus sonication	PCR Ct value
CF098	2	0	34.6
CF093	3	0	18.9
CF011	2	0	32.2
CF074	1	0	28.4
CF338	0	5	24.7
CF057	0	8	29.6
CF107	0	3	25.6
CF256	0	1	26.9
CF070	0	3	29.0
CF283	0	2	28.2
CF209	0	2	28.5
CF200	0	1	28.1
CF287	0	1	31.6
CF078	0	1	35.3
CF143	0	1	32.2
CF148	0	3	34.3
CF071	6	19	32.6
CF337	6	33	22.4
CF160	8	19	31.3
CF267	9	17	33.5
CF055	5	23	33.6
CF109	3	6	27.6
CF278	3	6	27.1
CF031	3	11	29.6
CF183	1	8	31.1
CF176	7	11	28.1
CF266	10	15	36.6
CF261	2	5	26.8
CF014	5	8	26.7
CF265	36	>100	25.5
CF163	6	8	30.2
CF175	1	1	31.2
CF320	1	1	33.2

It was noted that the two samples with a positive culture for *Penicillium* spp. were PCR negative. Given that the genus of *Penicillium* closely resembles that of *Aspergillus* it was expected that these may be PCR positive and indeed the manufacturer advises of cross reactivity with *Penicillium* spp. Further species identification of the *Penicillium* was not performed so it is unknown. PCR may have been negative for 2 reasons: each of the two samples only grew 1 CFU of *Penicillium* spp. so the copy number may have been too low to detect, secondly the MycAssay Aspergillus™ kit has only been shown to have definite cross reactivity with *Penicillium chrysogenum* and as speciation was not performed this may be the reason for negative PCR. Future experiments are needed to clarify the cross reactivity of this assay with different *Penicillium* spp.

The MycXtra™ DNA extraction kit uses bead beating to break fungal cell walls and release DNA. Different methods of fungal DNA extraction have been evaluated and bead beating is the most efficient and least time consuming (Griffiths et al., 2006). This kit also removes common inhibiting substances found in CF sputum such as tobramycin and DNase and is certified fungal DNA-free helping to reduce false positives, unlike some other kits which may be contaminated with fungal DNA (i.e. QIAamp DNA stool minikit) (Fredricks et al., 2005). Sonication and DNA extraction can be performed on 10 samples in under 3 h and are therefore suitable for use in high output microbiology laboratories.

Optimal fungal culture techniques have never been studied directly so there is little evidence to choose a single method to use as a gold standard against PCR. Sabouraud agar for *Aspergillus* species does appear to be the optimal growth medium (Bakare et al., 2003) and homogenisation is known to improve bacterial counts (Pye et al., 1995). However, the volume of sputum to culture and whether to concentrate organisms through centrifugation prior to agar plating is unknown. There is a wide variation in reported prevalence of *Aspergillus* in CF sputum from 5.6 to 57% (Bakare et al., 2003). This reflects variations in patient sample size, culture technique and region. Our positive culture rate was improved by sonication from 19% to 33% which

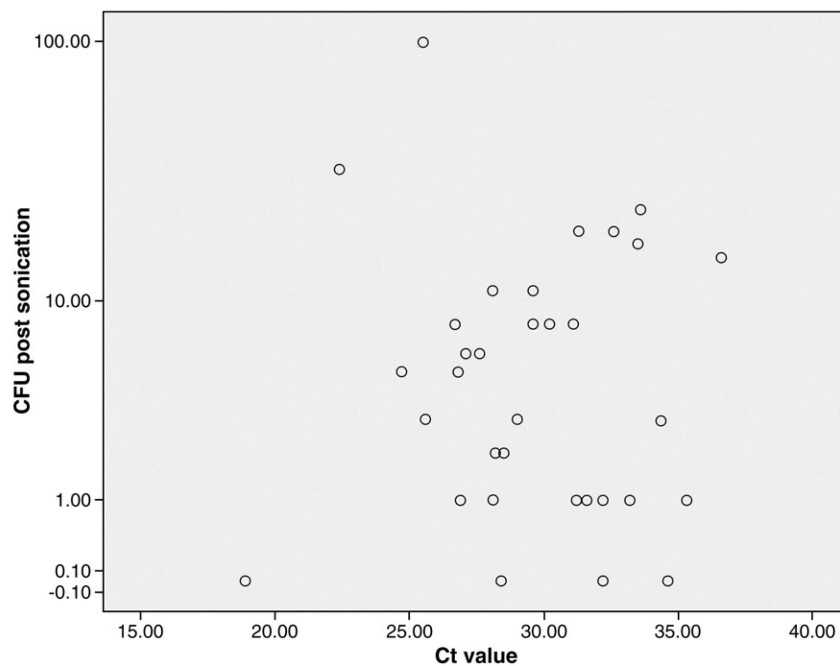

Fig. 4. Correlation between post-sonication CFU and PCR Ct value (r = −0.07).

appears average for UK studies in adults. The very high prevalence rates described by Nelson et al. (57%) and Schonheyder et al. (50%) (Nelson et al., 1979; Schonheyder et al., 1985) have not been reproduced in more recent studies. Despite this, PCR detected *Aspergillus* species in 73% of samples, more than any culture technique.

Overall, this study shows that PCR is significantly more sensitive than culture to detect *Aspergillus* in CF sputum but further studies are required to determine the clinical relevance of positive PCR in the context of disease phenotype. The increasing links between worse respiratory function and *Aspergillus* colonisation, with or without sensitisation in both asthma and CF, is suggestive of the importance of identifying *Aspergillus* in airway secretions (Amin et al., 2010; Denning et al., 2009; Fairs et al., 2010). It may also be important to determine the significance of the PCR results in the context of other markers of *Aspergillus* growth such as galactomannan. *Aspergillus* spores may simply be found adherent to CF sputum with no evidence of active growth and therefore play no role in disease. Furthermore, PCR is unable to distinguish between live and dead organisms. Galactomannan is found in the cell wall of *Aspergillus* and is released during growth. There are several studies which have detected galactomannan in BAL (Guo et al., 2010) and this could be extended to sputum. The complete liquification of CF sputum by sonication would allow galactomannan concentrations in sputum to be determined by ELISA as in BAL samples. Further studies are also needed to determine the repeatability of PCR within samples and over time and to determine the clinical significance of PCR Ct values.

In summary, sonication can be used effectively and efficiently to homogenise CF sputum in preparation for DNA extraction. It is superior to chemical homogenisation as it improves the sensitivity of DNA extraction. Further studies are now needed to determine the value of PCR in the diagnosis of *Aspergillus* related CF lung disease and in the monitoring of response to treatment.

5. Funding

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6. Potential conflicts of interest

David Denning holds founder shares in F2G Ltd. and Myconostica Ltd., both University of Manchester spin-out companies and has received grant support from F2G as well as the Fungal Research Trust, the Wellcome Trust, the Moulton Trust, The Medical Research Council, The Chronic Granulomatous Disease Research Trust, the National Institute of Allergy and Infectious Diseases, National Institute of Health Research and the European Union, AstraZeneca and Basilea. He continues to act as an advisor/consultant to F2G and Myconostica as well as other companies over the last 5 years including Basilea, Vicuron (now Pfizer), Pfizer, Schering Plough, Nektar, Daiichi, Astellas, Gilead and York Pharma. He has been paid for talks on behalf of Schering, Astellas, Merck, Dainippon and Pfizer. The other authors report no conflicts of interest.

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