marrow aspirate showed no evidence of haemophagocytic cells, but showed erythropoietic hyperplasia with dysplastic changes in erythroblasts. No evidence of a metabolic storage disorder was seen. Cerebrospinal fluid (CSF) showed no sign of inflammation, malignancy or evidence of haemophagocytic cells.

Analysis of peripheral blood cells (BD FACSCalibur system)[1] revealed increased CD4 T-cell count (2156 cells/μL; normal range: 1000–1800), reduced CD8 count (280 cells/μL, normal range: 800–1500) and high CD4:CD8 ratio (7.7; normal range: 1.0–1.6). There was a decrease in B-cell and natural killer (NK) cell counts. Other laboratory findings included a high ferritin level (2730 μg/L; normal up to 330) and increased triglycerides (2.9 μg/L; normal up to 1.7).

The perforin (*PRF1*) gene was amplified from extracted genomic DNA using a previously described method,[2,3,5] and polymerase chain reaction (PCR) products were sequenced (ABI3130 DNA sequencer, PE-Applied Biosystems, Foster City, CA). *PRF1* sequence analysis revealed the presence of homozygous c.1349C>T (T450M). Analysis of the parents' *PRF1* gene showed the same heterozygous mutation, although there was no family history of HLH.

The patient was referred subsequently to the bone marrow transplant unit for stem cell transplantation and was successfully transplanted with unrelated donor umbilical cord blood stem cells.

The pathogenesis of HLH remains controversial; however, uncontrolled inflammation reflected by T-cell and macrophage activation remains the hallmark of HLH.[4] Genetic studies of familial HLH reveals a link between mutations in the perforin (*PRF1*; MIM170280), *MUNC13-4* and *STX11* genes. Mutations in all thee genes have been found in up to 50% of familial HLH families.

Lee *et al*. investigated *PRF1* gene mutations in a cohort of 50 HLH families using direct sequencing. Overall, *PRF1* gene mutations were found in at least 50% of the families, with the mutations occurring anywhere in the *PRF1* gene.[5] Stepp *et al*.[6] sequenced the *PRF1* gene in eight unrelated HLH patients and found four with homozygous non-sense mutations and four patients with missense mutations.

The present study reports a homozygous mutation of the *PRF1* gene (MIM 170280). This mutation occurs in the EGF-like domain of the *PRF1* gene (exon 2) and was reported previously in a one-month-old female with HLH. That patient showed reduced NK cell activity (5%), reduced cytotoxic lymphocyte activity (41%), and reduced PRF1 protein activity shown by Western blotting.[7]

To the authors' knowledge, this is the first report of a *PRF1* gene mutation in a Saudi HLH patient. Diagnosis will enlighten and increase the awareness of such rare genetic diseases, especially in cases of consanguineous marriage. □

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Determination of optimum incubation time for release of bacteria from sputum of patients with cystic fibrosis using dithiothreitol (Sputasol)

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Cystic fibrosis (CF) is the most common inherited fatal disease in persons originating from a Caucasian and European background, and currently affects approximately 8000 individuals in the UK.[1] The defective gene carrying the mutation is carried by approximately one in every 25 people in the UK population. This means that more than two million people in the UK are symptomless carriers of the defective gene.[1]

Cystic fibrosis is an autosomal recessive condition whereby two alleles carrying a polymorphism in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene

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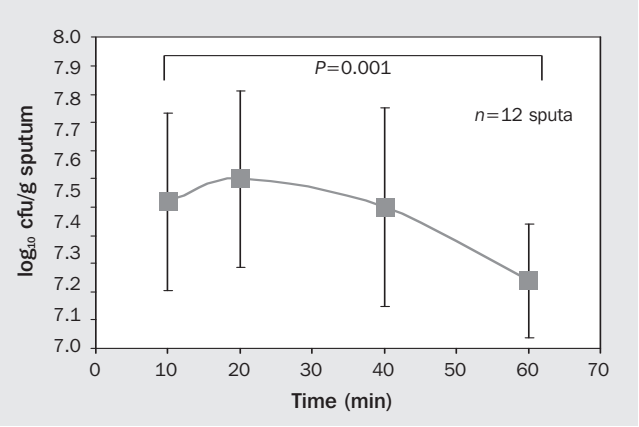

Fig. 1. Time course (to 60 min) showing release of total culturable microorganisms from adult CF sputa (12 patients) in the presence of dithiothreitol (Sputasol) at 37°C.

phenotypically manifest the disease state through a variety of multi-organ problems associated with a pharmacological dysfunction to regulate sodium and chloride secretion across cell membranes. The most common complication of CF is the recurrence of chronic chest infections usually caused by bacterial pathogens.[2] Cystic fibrosis patients continue to suffer from recurrent and chronic respiratory tract infections and most of their morbidity and mortality is due to such infections throughout their lifetime.[3]

These infections are usually dominated by Gram-negative organisms, especially by the pseudomonads, including *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex and *Stenotrophomonas maltophilia*. Dysfunction of *CFTR* results in the production of mucoviscous sputum with unusual rheological properties, which is difficult to expel from the airways due to its composition as well as mucocillary dysfunction. These events result in the accumulation of viscous sputum, mainly composed of bacterial glycocalyx, mainly polysaccharide, which traps infecting bacteria within and acts as a physical barrier to the movement of agents across the matrix, including the uptake of adenoviral vectors delivering gene therapy nucleic acid.[3] Hence, it is important that this glycocalyx matrix is broken down fully to release bacterial flora into the extracellular area during any subsequent microbiological investigation of CF sputa.

Although previous studies have evaluated the application of the mucolytic agent dithiothreitol (also known as Cleland's reagent) for the liquification of sputum from patients with CF,[4,5] there has not, as yet, been any scientific evidence-base to define the optimum time for the treatment of sputum with this agent, in order to release the maximum number of bacterial cells for downstream identification and counting purposes. Such data are also not available from the literature or from the manufacturers of dithiothreitol, resulting in employment of non-standardised assays, whereby laboratories decide on time of incubation empirically and individually.

Many clinical microbiology laboratories supporting CF units are today processing a greater number of specimens, due to rationalisation of microbiology services. Therefore, it is the primary aim of this study to define the optimum incubation time for dithiothreitol in CF sputum, to permit the release of the highest number of organisms. This will allow CF microbiology laboratories an evidence-base to define their standard operating procedure (SOP) for sputum processing.

Fresh expectorated sputum (5–10 g/patient) was collected, following physiotherapy, from 12 adult patients with CF, who were in-patients at the Adult Regional CF Unit, Belfast City Hospital, during the summer of 2008. All patients had a well-characterised diagnosis of CF and were chronically infected with *P. aeruginosa*. Sputum was collected immediately after a standardised session of physiotherapy, was stored at ambient temperature and was processed within 4 h of collection. Dithiothreitol (Sputasol) was reconstituted from a commercial aliquot (Oxoid SR089A, Oxoid, Poole, England) to give a final concentration of 100 µg/mL. Fresh sputum (1 mL/min) from individual CF patients was mixed with an equal volume (1:1) of a solution of dithiothreitol and was incubated at 37°C for 10 min, 20 min, 40 min and 60 min, before further processing and enumeration. Serial dilutions of sputum were prepared in quarter-strength Ringer's solution diluent (Oxoid BR52).

From the 10^{-4}, 10^{-5} and 10^{-6} dilutions in triplicate, 100 µL inoculum was spread on the surface of Columbia Agar Base (Oxoid CM331) supplemented with 5% (v/v) defibrinated horse blood (E&O Laboratories, Bonnybridge, Scotland) and incubated at 37°C for 48 h prior to counting. All cultured flora, regardless of colonial morphology and appearance, were enumerated and the total viable count (TVC) was expressed as $\log_{10}$ colony-forming units (cfu) per gram original sputum.

Mean results of sputum from 12 CF patients showed that initially the culturable counts in the sputum ranged from 7.03 to 7.83 log cfu/g, with a mean count of 7.52±0.31 log cfu/g sputum. The maximum count of total culturable microorganisms in sputum was achieved 20 min after the addition of Sputasol, after which time the enumerative counts began to decrease (Fig. 1). Statistically, counts were

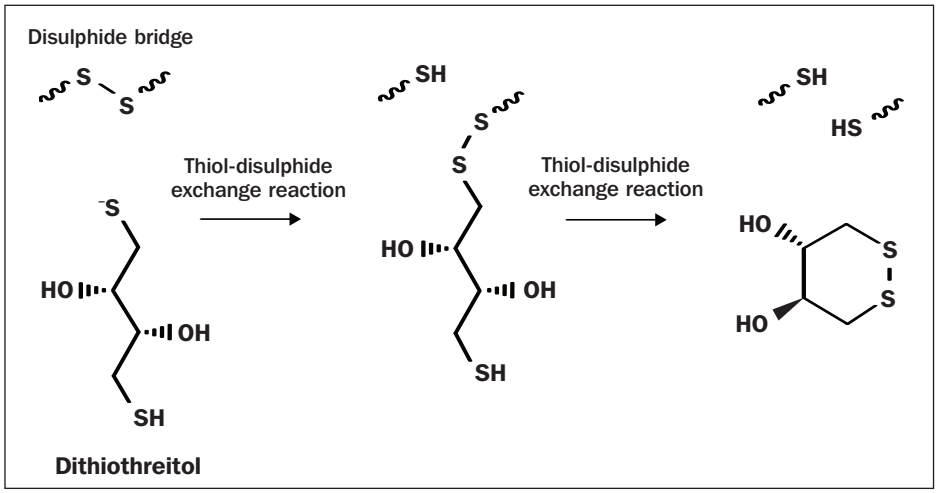

Fig. 2. Chemical reduction of disulphide bonds with dithiothreitol.

significantly different between 10 min and 60 min incubation at 37°C ($P=0.001$; *t*-test).

Dithiothreitol ($C_4H_{10}O_2S_2$; [2S,3S]-1,4-Bis-sulfanylbutane-2,3-diol) is a strong reducing agent, due to its high conformational propensity to form a six-membered ring with an internal disulphide bond. The viscosity of CF sputum is partially due to the presence of strong –SH and S-S linkages in the glycoprotein matrix, which can be reduced by the presence of dithiothreitol. The reduction of a typical disulphide bond proceeds by two sequential thiol-disulphide exchange reactions (Fig. 2). The intermediate mixed-disulphide state is unstable because the second thiol of dithiothreitol has a high propensity to close the ring, forming oxidised dithiothreitol and leaving a reduced disulphide bond.

As bacteria are not uniformly distributed in sputum,[6] it is important that subsequent analysis is performed on a specimen that is in as homogeneous a state as possible. This can be aided by physical homogenisation of sputum or the addition of liquefying agents to sputum to degrade –SH and –SS bonds. Microbiology laboratories supporting CF units historically have employed either N-acetyl-cysteine or dithiothreitol as a mucolytic agent to liquefy CF sputum, prior to downstream qualitative and quantitative assays. However, Shah and Dye[4] have demonstrated the superior properties of dithiothreitol against N-acetyl-cysteine, in terms of microbiological release of organisms from sputum. In the seminal publication on dithiothreitol as a mucolytic agent of CF sputum by Hammerschlag *et al.*,[5] the authors showed that dithiothreitol aids sputum analysis by releasing bacteria for enumeration. However, they failed to address the importance of incubation time, selecting an arbitrary time of 15 min for their experiments.

In the absence of an evidence-based approach to the laboratory processing of sputum in relation to this specific issue, busy laboratories may incubate their specimens for an insufficient period of time, due to space and time constraints. This results in a low yield of the total microbial population trapped within the sputum matrix, with downstream consequences in terms of the quality of service offered.

In conclusion, this small study demonstrates that the optimum incubation time for dithiothreitol is 20 min (37°C), which permits the maximum release of microorganisms from CF sputum. Clinical microbiology laboratories should now consider amending their individual SOPs to incorporate this datum point, in order to maximise microbial cell release and optimise laboratory processing/handling of CF sputum specimens. □

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