# *Helicobacter pylori* infection in children: association with giardiasis

M. MONAJEMZADEH[*], M. T. H. ASHTIANI[*], A. M. ALI[*], M. N. SANI[*], S. SHAMS[*], N. KASHEF[†], R. SHAHSIAH[‡] and N. ERAM[*]

[*]*Children's Medical Centre Hospital, Tehran University of Medical Sciences;* [†]*Department of Microbiology, Faculty of Biology, University of Tehran; and* [‡]*Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran*

*Helicobacter pylori* infection is one of the most important factors in the pathogenesis of upper gastroduodenal disease.[1] One of the main risk factors for *H. pylori* is low socioeconomic status. Crowding and markers of poor hygiene in childhood are strong risk factors, and infection is now thought to be transmitted by several mechanisms involving the faecal-oral and oral-oral pathways.[2,3]

*H. pylori* and giardiasis are two common causes of recurrent abdominal pain, both of which are common in young children in Iran; however, very few reports mention co-infection with giardiasis and *H. pylori* in children.[4,5] Thus, this small study aims to evaluate the frequency of *H. pylori* infection in children infected with *Giardia lamblia*, an intestinal parasite known to be acquired by the faecal-oral route.

A case-control study of children with and without giardiasis was conducted over a six-month period (February to July 2008). Forty-two children under the age of 14 were enrolled in each group, with children not infected with *G. lamblia* being the control group. The protocol was approved by the Institutional Review Board of Tehran University of Medical Sciences, and written informed consent was obtained from the parents of all children prior to enrollment in the study. A crowding index was calculated based on that described by Moreira *et al*.[4] Briefly, the number of household members was divided by the number of rooms in the house. Both groups comprised patients attending for routine general health evaluation with no history of chronic or acute disease (eg immunodeficiency, neoplasm) and had not received antibiotic or antacid treatment in the previous eight weeks.

The presence of *G. lamblia* was evaluated by parasitological evaluation of stool samples. *H. pylori* status was determined by antigen detection in stool samples in an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (HpSA ELISA; Astra, Italy), following the manufacturer's instructions.

Statistical analysis was performed using SPSS, version 13 (SPSS, Chicago IL, USA). The relationship between the presence of giardiasis and *H. pylori* infection was evaluated by the $\chi^2$ test and odds ratio (OR).

*Correspondence to: Maryam Monajemzadeh*
*Pathology Ward, Children's Medical Centre Hospital*
*Keshavarz Boulevard, Tehran, Iran*
*Email: monajemz@sina.tums.ac.ir*

Table 1 shows the characteristics of the case and control groups. Of the 82 subjects studied, 23 (27.4%) were found to have *H. pylori* antigen. Of these, 17 (73.9%; six boys, 11 girls) were in the case group, and six (26.1%; two boys, four girls) were in the control group. The frequency of *H. pylori* infection in the case group was greater than that in the control group ($P$=0.007).

No significant relationship between age and *G. lamblia* infection was found. The results also indicated that gender, number of rooms, and crowding index were not associated with *G. lamblia* infection. The number of household subjects with giardiasis was greater than those without *G. lamblia* infection ($P$=0.036); however, home area of those in the latter group was larger ($P$=0.001).

After adjusting for selected covariates using logistic regression, the only variable that remained independently associated with *H. pylori* positivity was the presence of *G. lamblia* in faeces. The presence of *G. lamblia* significantly increased the odds of *H. pylori* infection by 4.5. Age, gender and crowding index were not significantly associated with *H. pylori* infection.

If the faecal-oral route is significant in the transmission of *H. pylori*, one would expect the prevalence of this bacterium to correlate with that of *G. lamblia*. Statistical analysis of the data presented here showed significant correlation between giardiasis and *H. pylori* infection; results that concurred with those of Moreira *et al*.[4]

*H. pylori* infection predisposes patients to ulcers and hypochlorhydria. Decreased gastric acidity is a prerequisite for localisation of *G. lamblia* to the gastric mucosa, as it is known that hydrochloric acid acts as a chemical barrier to microbes.[6]

This association between *H. pylori* infection and giardiasis may have several clinical suggestions in regard to the routes of transmission, the possibility of a synergy in metronidazole resistance, and a common pathogenesis scenario, leading to gastrointestinal metaplasia.

The findings of this small study indicate that *H. pylori* should be searched for carefully when examining stool samples that show giardiasis, and trophozoites of *G. lamblia* should be searched for when examining gastric biopsy

**Table 1.** Characteristics of subjects with and without giardiasis.

| | Giardiasis | | *P* value |
|---|---|---|---|
| | Positive ($n$=42) | Negative ($n$=42) | |
| Age (years; mean±SD) | 5.7±2.8 | 6.1±3.7 | 0.57 |
| Gender (male; %) | 18 (42.9) | 24 (57.1) | 0.19 |
| Number in household (mean±SD) | 4.3±1.1 | 3.8±1 | 0.036 |
| Number of rooms (mean±SD) | 2.2±0.8 | 2.2±0.7 | 0.889 |
| Home area ($m^2$; mean±SD) | 75.7±19.9 | 92.6±22.7 | 0.001 |
| Home area/number in household | 18.8±7.3 | 25.5±8.2 | <0.001 |
| Crowding index (%) | | | 0.535 |
| ≤1 | 6 (14.3) | 7 (16.7) | |
| 1.1–2 | 20 (47.6) | 25 (59.5) | |
| 2.1–3 | 10 (23.8) | 5 (11.9) | |
| >3 | 6 (14.3) | 5 (11.9) | |
| *H. pylori* positivity (%) | 17 (73.9) | 6 (26.1) | 0.007 |

specimens showing chronic atrophic gastritis. Further studies to investigate this relationship in more detail are required. □

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# Molecular conservation within LES9F and PS21 Liverpool epidemic strain (LES) markers in wild-type clinical *Pseudomonas aeruginosa* isolated from the sputum of adult patients with cystic fibrosis


Y. KAKINUMA[*,†], C. E. GOLDSMITH[*], A. WATT[‡], J. S. ELBORN[§,#], Y. MAEDA[*,¥], J. C. RENDALL[§], V. HALL[§], J. McCAUGHAN[‡], A. REID[¶], B. C. MILLAR[*], M. MATSUDA[†] and J. E. MOORE[*,¶]

[*]*Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast, Northern Ireland;* [†]*Laboratory of Molecular Biology, School of Environmental Health Sciences, Azabu University, 1-17-71 Fuchinobe, Sagamihara, Kanagawa, Japan;* [‡]*Department of Medical Microbiology, Kelvin Building, The Royal Group of Hospitals, Belfast;* [§]*Regional Adult Cystic Fibrosis Unit, Belfast City Hospital, Belfast;* [#]*Department of Respiratory Medicine, The Queen's University of Belfast, Respiratory Medicine, Belfast City Hospital, Belfast;* [¥]*School of Biomedical Sciences, Centre for Molecular Biosciences, University of Ulster, Coleraine; and* [¶]*The Royal Belfast Hospital for Sick Children, Royal Group of Hospitals, Grosvenor Road, Belfast, Northern Ireland.*


The most common complication of cystic fibrosis (CF) is the recurrence of chronic chest infections usually caused by bacterial pathogens. 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, which are usually dominated by Gram-negative organisms, especially *Pseudomonas aeruginosa*, as well as members of the *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia* and several emerging Gram-negative organisms.[1] One main clinical aim in their management is the prevention of acquisition and subsequent progression of bacterial respiratory pathogens to the chronic stage, as such events lead to a declining cascade of infection and inflammation, resulting in decreasing lung function and premature death.


*Correspondence to: Professor John E. Moore, Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 7AD, Northern Ireland, UK*
*Email: jemoore@niphl.dnet.co.uk*


Recently, several reports have described the emergence of the Liverpool epidemic strain (LES) of *P. aeruginosa* in CF patients.[2] This LES strain has been reported to be the most frequently isolated clone obtained from CF patients in England and Wales.[3] This epidemic strain has also been reported to cause superinfection[4] and is associated with greater morbidity in patients than seen with other non-LES *P. aeruginosa* strains.[5] In addition, it has been demonstrated to be highly transmissible from a CF patient to non-CF parents,[4] while there has also been a recent report of transmission from a CF patient to a cat,[6] which resulted in increased morbidity for recipients of LES *P. aeruginosa.*

As a result of this, attention has been directed to the development of reliable molecular diagnostic assays to differentiate LES *P. aeruginosa* from non-LES *P. aeruginosa* and several methods have been published, including the LES9F and PS21 markers as target amplicons for the presence of LES.[7] The recent publication of the entire genome of an LES-positive strain (*P. aeruginosa* LESB58; GenBank accession number: FM209186) has allowed comparison of clinical wild-type isolates to LESB58.

However, what remains unclear is the amount of variation that exists within these target LES amplicons and how conserved they are in wild-type *P. aeruginosa* isolates from CF patients, as presently there is only one gene sequence of these loci in GenBank (submitted in early January 2009). Hence, this short sequencing study aims to estimate the degree of genetic hypervariability that exists within wild-type LES *P. aeruginosa* organisms obtained from the sputum of adult CF patients and LESB58.

Examples of LES *P. aeruginosa* were isolated from the sputum of six randomly selected adult patients attending the Northern Ireland Regional Adult Cystic Fibrosis Centre, Belfast City Hospital, who were known to be LES-positive patients. The LES status was checked through the development of a multiplex assay of three targets (PS21,[7] LES9F[7] and FpvAIII pyoverdine[8]). Only *P. aeruginosa* isolates that were concurrently positive for all three amplicons were selected for subsequent dideoxy sequencing, as described previously.[9] Within each target, resulting sequences from all isolates were aligned with each other, as well as with LESB58 (Table 1). From the current study, a representative sequence of LES PS21 and LES 9F has been submitted to GenBank with the respective accession numbers FJ710791 and FJ710792. Sequencing results from the *P. aeruginosa* isolates were totally conserved within the entire PS21 amplicon, as well as having total similarity with this region in LESB58, despite several mutations being detected within the FpvAIII pyoverdine with these isolates (data not shown). In addition, all the isolates

**Table 1.** Comparison of LES PS21 and LES 9F in wild-type *Pseudomonas aeruginosa* isolated from CF patients and the reference strain LESB58 (GenBank Accession No: FM209186).

| Target | Submitted GenBank Accession Number | Closest BLAST sequence match | Similarity | Position (in relation to FM209186) |
|---|---|---|---|---|
| LES PS21 | FJ710791 | *Pseudomonas aeruginosa* FM209186; PLES_26321 | 100% | np 2833095–2832811 |
| LES 9F | FJ710792 | *Pseudomonas aeruginosa* FM209186; PLES_23591 | 99% | np 2524896–2524524 |

demonstrated a single nucleotide deletion of an adenine base at position 2524879 (FM209186) of the LES 9F gene locus.

This small study demonstrates that LES PS21 and LES 9F are highly conserved in the wild-type CF *P. aeruginosa* isolates examined, even in the presence of several mutations in the pyoverdine gene locus. BLAST analysis of these sequences demonstrates the uniqueness of these sequences in nature, whereby only one match was obtained (GenBank accession number: FM209186). At this stage, the significance of this is unclear, but it is important to be able to note variation within these amplicons and possible variations in clinical disease states. Further analysis is now required of isolates from around the world, in order to examine potential geographical diversity. □

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# *PRF1* gene mutation in a Saudi patient with haemophagocytic lymphohistiocytosis

M. A. AL BALWI[*,†,§], T. AL HARBI[*,‡], I. AL ABDULKAREEM[*,§] and A. H. HAJEER[*,†]

[*]*King Saud bin Abdulaziz University for Health Sciences, College of Medicine;* [†]*Department of Pathology and Laboratory Medicine, and* [‡]*Department of Pediatric Medicine, King Abdulaziz Medical City; and* [§]*King Abdullah International Medical Research Centre, Molecular Biology, King Abdulaziz Medical City, Riyadh, 11426 Saudi Arabia*

Haemophagocytic lymphohistiocytosis (HLH) is a rare autosomal inherited disease associated with activated macrophages that engulf erythrocytes, leucocytes, platelets and their precursor cells in bone marrow, lymph node, spleen and other tissues.[1,2] Patients with HLH manifest cellular immunological dysfunction of regulatory pathways that normally terminate in effector immune responses.[3]

Hereditary and sporadic cases of HLH have been reported mainly in children,[5] although the condition can affect other age groups.[6–9] The incidence of HLH is estimated to be 1.2 per 1,000,000.[4–6] Clinical manifestations include decreased fetal activity, neonatal hypotonia, neonatal feeding difficulties, hyperphagia with obesity, hypogonadism, short stature, small hands and feet, characteristic facial features, and mild to moderate mental retardation.

Haemophagocytic lymphohistiocytosis may be familial, or associated with a number of different infections, autoimmune disorders, or may occur together with malignancy.

The case present here is of an 18-month-old boy born at 34 weeks' gestation to a young couple (first cousins). Written informed consent was obtained from the parents of the patient for publication of this case report. The pregnancy suffered premature rupture of the membrane and neonatal polycythemia that required partial exchange of blood.

At the age of seven weeks the child was admitted to the paediatric intensive care unit with suspected septic shock syndrome. All microbiological tests were negative. Physical examination revealed hepatosplenomegaly and blood tests showed neutropenia (absolute neutrophil count: 400 cells/μL), thrombocytopenia (platelet count: 23,000/μL) and anaemia (haemoglobin [Hb]: 55 g/L). The patient recovered and his blood counts showed partial improvement.

At the age of three months he was admitted to the paediatric ward with fever and low blood counts that required frequent blood and platelet transfusion. Bone

*Correspondence to: Dr M. Al Balwi*
*Email: balwim@ngha.med.sa*

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)


M. McCLEAN[*,†], T. STANLEY[*], C. E. GOLDSMITH[‡], B. C. MILLAR[‡], B. McCLURG[‡], J. S. ELBORN[§,#], C. J. LOWERY[†], J. S. G. DOOLEY[†], J. C. RENDALL[§] and J. E. MOORE[†,‡]

[*]*Northern Ireland Mycobacterium Reference Laboratory, Department of Medical Microbiology, The Royal Group of Hospitals, Belfast;* [†]*School of Biomedical Sciences, University of Ulster, Coleraine, Co. Londonderry;* [‡]*Northern Ireland Public Health Laboratory, Department of Bacteriology;* [§]*Northern Ireland Regional Adult Cystic Fibrosis Unit; and* [#]*Respiratory Medicine Research Group, Centre of Infection & Immunity, Queen's University, Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, Northern Ireland, UK*


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


*Correspondence to: Professor John E. Moore*
*Northern Ireland Public Health Laboratory, Department of Bacteriology*
*Belfast City Hospital, Belfast BT9 7AD, Northern Ireland, UK*
*Email: jemoore@niphl.dnet.co.uk*

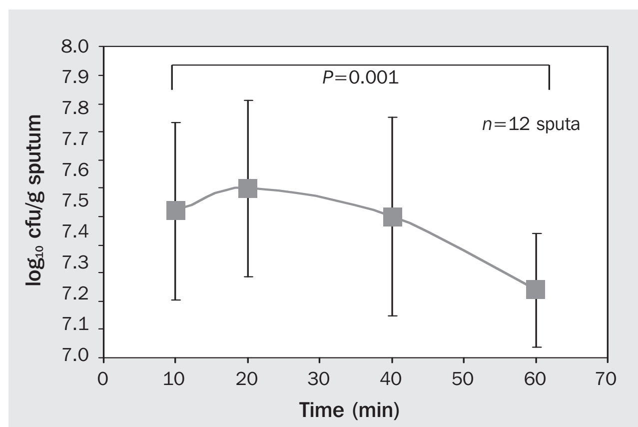


**Fig. 1.** Time course (to 60 min) showing release of total culturable microorganisms from adult CF sputa (12 patients) in the presence of dithiothreitol (Sputasol) at 37°C.

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

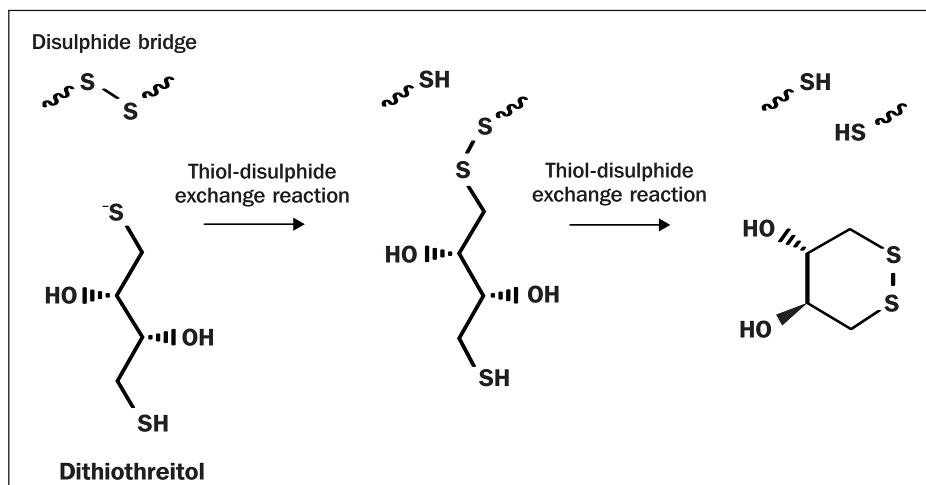


**Fig. 2.** Chemical reduction of disulphide bonds with dithiothreitol.

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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