results. Serial dilution of stained material (to 10,000-fold) may allow such material to be amplified, and this dilution stage should be incorporated into molecular diagnostic standard operating protocols.

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Molecular detection of *Haemophilus influenzae* in COPD sputum is superior to conventional culturing methods

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Chronic obstructive pulmonary disease (COPD) is predominantly a disease of smokers and ex-smokers that not only causes significant morbidity and mortality but also has a strong impact on the quality of life of affected individuals.[1] The disease is characterised by airway inflammation and varying degrees of airflow limitation resulting in irreversible lung damage. Symptoms include breathlessness, coughing, wheezing and sputum production. Disease progression is characterised by increasing disability due to increasing breathing problems, hospital admission and premature death.[2] Unlike the declining mortality associated with other diseases (e.g., cardiovascular disease and stroke), the mortality rate in COPD is actually increasing.[3]

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In COPD the lower airways may be persistently colonised by microbial pathogens (isolated from sputum and bronchial lavage [BAL] samples), even during stable disease. The organisms most commonly isolated are *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*,[4,5] and all are associated with increased inflammation and lung damage.[6] Of these, *H. influenzae* is the most frequently identified[7] and the most common cause of COPD exacerbations.[8,9] In a recent study, it was demonstrated that *H. influenzae* bronchial colonisation in COPD patients was associated with sputum neutrophilia, an identifiable exaggerated inflammatory response, and a significant decline in lung function. In contrast, colonisation with *H. parainfluenza* was not associated with an inflammatory response, suggesting that this organism may not worsen disease severity,[6] even though colonisation has been reported in 20% of COPD patients.[9]

Despite frequent carriage of *H. influenzae*, long-term use of prophylactic antibiotics is not currently recommended as a preventative treatment option[1,10] even though studies demonstrate an associated small decrease in days of illness if used.[11] Concerns regarding the impact of long-term antibiotic use on patient health and the development of antibiotic resistance are thought to outweigh any small benefit. Therefore detection of *H. influenzae* in COPD sputum samples using conventional culturing continues to guide antibiotic usage, and rapid and sensitive molecular detection would allow a prompt clinical response that would greatly improve the quality of patient life and may slow progression of the disease.

Twenty COPD patients were recruited from the Launceston General Hospital with a diagnosis of COPD, respiratory symptoms (e.g., breathlessness, cough, sputum), a smoking history of ≥10 pack years (where one pack year is 20/day for one year or the equivalent number of cigarettes), an FEV1:FVC ratio after bronchodilation of <0.7, and one to two hospital admissions for COPD in the last year. Once enrolled in the study, patients provided a sputum sample (either spontaneously or using a standard saline inhalation procedure[12]) and another sample six months later or upon admission for an acute exacerbation. All patients enrolled in the study gave written informed consent. The study protocol was approved by the Tasmania Human Research Ethics Committee (H0009201).

A total of 36 sputum samples were collected and immediately stored at 4°C before being weighed and then homogenised at 37°C with four volumes of 10% Sputolysin (Calbiochem) for 30 min and plated on chocolate agar supplemented with bacitracin and incubated at 37°C anaerobically for 48 h. Isolates consistent with *H. influenzae* morphology (1–2 mm diameter, flattened convex with or without sunken centres, smooth, entire edge, translucent, greyish) were spread over tryptone soy agar plates with disks of X factor (hemin), V factor (NAD or NADP) and X+V factors, and plates were then incubated as described above. Isolates with growth around the X+V factor only were designated *H. influenzae* and stored. Genomic DNA for the polymerase chain reaction (PCR) assay was extracted from the remaining sputum samples using the method of Reischl *et al*.[13]

The use of 16S ribosomal DNA, which has been used for *H. influenzae* identification,[14] fails to provide sufficient differentiation from *H. parainfluenzae*.[15] The widely used outer membrane protein (OMP) P6 PCR method detects *H. influenzae* and other *Haemophilus* spp.,[15] and is only able to differentiate *H. influenzae* from *H. parainfluenzae* when utilised in a more complex and expensive PCR (e.g., loop-mediated isothermal amplification [LAMP] assays[15] or probe-based PCR[16]). Therefore, this study used the gene encoding OMP4 (Genbank Accession No CP000057) that displays the appropriate amount of conservation for species-level identification for *H. influenzae*. Primers were designed using all available Genbank sequences of *H. influenzae* and *H. parainfluenzae* and tested against 16 strains of *H. influenzae* and six strains of *H. parainfluenzae*. Primers were 100% specific for *H. influenzae* detection (OMP4–425F: 5′- ACGTAACAAACCGCAAAGACAG -3′ and OMP4–504R: 5′-TTCCACGCCATTGAAACCTAAG-3′). All PCRs were performed in duplicate using Platinum *Thermus aquaticus* (*Taq*) DNA polymerase (Invitrogen), according to the manufacturer's instructions. Reactions were run on the Rotor-Gene 3000 thermocycler under the following conditions: 50˚C for 2 min, 95˚C for 2 min, 40 cycles of 95˚C for 15 sec, 62˚C for 30 sec and 72˚C for 15 sec, and 95˚C for 1 min and 50˚C for 1 min. The genomic DNA samples from the 36 sputum isolates were tested by PCR for the presence of *H. influenzae* and the products electrophoresed on 3% agarose gel stained with ethidium bromide. All band sizes were as expected (80 bp) and DNA sequencing confirmed amplification of OMP4.

Conventional culture detected *H. influenzae* in five of the 36 sputa (14%). The PCR assay was more sensitive and detected *H. influenzae* in the same five samples, as well as nine additional sputum samples (39% in total). Similar findings were reported by Murphy *et al.* who reported *H. influenzae* DNA to be present in sputum samples that yielded negative cultures.[4] In a study analysing cerebrospinal fluid (CSF), *H. influenzae* was cultured from 28.6% of samples, but by molecular methods (using 16S) this increased to 45.2% of samples.[14] Therefore, COPD sputum culturing significantly underestimates the frequency of *H. influenzae* colonisation and this may have an impact on our understanding of bacterial colonisation in COPD, and could disadvantage patients who would benefit from antibiotic therapy.

The method developed here differs from other published methods for molecular detection of *H. influenzae* in biological samples in several ways. Other methods are either based on strain-specific detection, fail to differentiate *H. influenzae* and *H. parainfluenzae* or require multiple PCR reactions.[14,17,18] Therefore, the current method has the advantage of specifically detecting *H. influenzae* in a single reaction.

Conventional culture-based detection of *H. influenzae* is time-consuming (up to a week) compared to molecular detection, which has the advantage of being rapid, sensitive and cost-effective, as samples can be analysed in a single day. Rapid detection could aid clinical decisions for patients with severe COPD who are exacerbating, and in the management of COPD patients with long-term moderate disease. Antimicrobial treatment in COPD tends to focus on treatment of patients with severe disease undergoing exacerbation.[7] In these patients, sputum microbiology is performed routinely and is a significant burden (i.e., time and cost) in microbiological laboratories.[19] The use of PCR analysis could save heathcare resources.

Recent evidence also suggests that a significant proportion of COPD patients with mild, stable disease demonstrate long-term infection with *H. influenzae* and this colonisation is specifically associated with neutrophilia, increased inflammation, and reduced lung function. The molecular method presented here suggests that *H. influenzae* infection may be up to three times as common as previously thought in COPD, which has significant implications as it is in this group of patients that therapeutic interventions are expected to provide maximal impact in terms of reducing future disability and acute healthcare utilisation.[6] A rapid, cost-effective and sensitive method for detecting *H. influenzae* therefore has the potential to improve health and slow disease progression.

In conclusion, the use of a sensitive molecular assay reveals that conventional microbiological methods underestimate COPD *H. influenzae* colonisation by one-third. This rapid, sensitive and cost-effective method for differentially detecting *H. influenzae* (from *H. parainfluenzae*) could help clinicians treat COPD patients effectively, especially those who have moderate disease or are experiencing an exacerbation of their condition.

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Rapid progression to diabetes in a four-year-old girl with cystic fibrosis

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Cystic fibrosis (CF) results from mutations in the gene that encodes the CF transmembrane conductance regulator protein located on chromosome 7. This gene encodes a protein that functions as a cyclic adenosine monophosphate-regulated chloride channel. Abnormal function of the channel results in aberrant conductance across the apical membrane of epithelial ductal cells in various organs (e.g., lung, pancreas, sweat gland, liver, nasal mucosa, salivary gland and colon).[1] The usual clinical presentation of CF includes persistent and recurrent pulmonary infection as well as symptoms of pancreatic insufficiency. Cystic fibrosis-induced diabetes can occur at any age, with mean onset reported to be 18–21 year.[2]

Although CF–related diabetes (CFRD) usually presents in the second decade of life, it has been reported in children as young as 10 years.[3] This study describes early-onset diabetes in a four-year-old child with CF complicated with recurrent lung infections that resolved after insulin therapy.

The patient was diagnosed with CF at the age of two after presenting with failure to thrive and respiratory infection. The sweat test revealed sweat chloride concentrations of 85 mmol/L and 72 mmol/L (0–40 mmol/L) in consecutive samples. As genotyping was not available, diagnosis of CF was based on clinical and laboratory values. After implementing substitutional pharmacotherapy in the form of pancreatic enzyme supplementation and inhaled corticosteroid therapy, the patient was in good health. She had a neonatal history of pulmonary artery stenosis and balloon pulmonary angioplasty, and subsequent endovascular stent implantation.

After two uneventfully years, she was hospitalised for recurrent lung infections resistant to antibiotics (teicoplanin and meropenem). Despite high calorific nasogastric and oral feeds, she failed to meet her goal weight. The patient weighted 13 kg (<3rd percentile) and measured 96 centimeters in height (<3rd percentile). On physical examination, she showed respiratory distress in the form of tachypnoea and retractions of the intercostal muscles. Breathing was deep and rapid, while chest auscultation revealed symmetric moderate aeration with crackles, rhonchi and wheezes. Her transcutaneous oxygen saturation was 94% while receiving 6 L/min nasal oxygen. Other systemic examinations were normal. Chest radiograph revealed parenchymal nodular opacities with prominent upper lobe bronchiectasis, consistent with CF (Fig 1a). A computed tomography (CT) scan of the thorax revealed bilateral perihilar consolidation.

Laboratory studies revealed high blood glucose level (412 mg/dL), low serum insulin level (1.64 µiu/mL) with low C-peptide (0.54 ng/mL [0.9–4.2]) level. Her HbAlc was 10.1%. Sedimentation rate was 35 mm/h and C-reactive protein level was 77 mg/dL. Arterial blood gas values showed pH 7.35 and a bicarbonate of 15 mmol/L. Urine and serum ketones were negative. Islet cell antibodies, glutamic acid decarboxylase antibodies and urinary ketones were negative. Subcutaneous insulin was initiated and treatment with benzylpenicillin for 14 days. Her laboratory data and radiological abnormalities markedly improved (Fig 1b). The remainder of the hospital course focused on insulin adjustment and diabetes education for the patient and family. The patient was discharged on multiple daily injections of insulin (0.4 units/kg/day) and pancreatic enzyme supplementations, and her subsequent growth rate improved. At follow-up visits, the patient demonstrated consistent and improved weight gain. At six years of age, she required 0.6 units/kg/day of insulin and had an HbA1c of 7.2%.

As patients with CF are now living longer because of improved medical care, CFRD has become the leading co-

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