

# Hydrogen cyanide emission in the lung by *Staphylococcus aureus*

To the Editor:

Early detection of *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients is crucial, since eradication in a later stage is extremely difficult [1]. Until now, it has been assumed that hydrogen cyanide (HCN) can be considered as a specific biomarker for *P. aeruginosa* in exhaled breath [2–5]. Only *Burkholderia cepacia* is also known to produce HCN *in vitro* [6, 7], but HCN is not a biomarker for *B. cepacia* complex infection *in vivo* [8]. To our knowledge, actual proof that HCN is not produced by other pathogens is lacking. Investigations into the emission of HCN by *Staphylococcus aureus*, a predominant CF pathogen throughout childhood, have been very limited. In a single report, five strains have been shown to produce low values *in vitro* [9], and *in vivo* studies have not been reported.

We therefore measured the real-time HCN production by clinical *S. aureus* isolates *in vitro*, and HCN levels in the exhaled breath of *S. aureus*-infected CF patients, in comparison with non-infected CF patients, as well as healthy persons as controls. Furthermore, we investigated whether the *S. aureus* strains cultured from patients in whom HCN was detected in breath, were capable of producing HCN.

Five clinical isolates of *S. aureus* from sputum of CF patients were inoculated into 50-mL brain-heart infusion broth in an initial concentration of  $5 \times 10^6$  CFU·mL<sup>-1</sup>. The setup used for analysing bacterial headspace (in triplicate) and determination of growth curves has been previously described.

CF patients (aged  $\geq 6$  years old) were recruited from the outpatient clinic of Radboud University Medical Center and Erasmus Medical Center (Nijmegen and Rotterdam, the Netherlands, respectively). This study was approved by the local medical ethics committee (CMO – Nijmegen/Arnhem) which waived written informed consent. Oral consent was obtained from participants aged  $\geq 12$  years and from parents of children aged 6–18 years.

We included nonsmoking, infected and non-infected CF patients and healthy controls. Infection was defined as culturing *S. aureus*, *P. aeruginosa* or *B. cepacia* in sputum on the day of breath sampling or in  $>50\%$  of sputum samples (minimum of four samples) over the previous 12 months. Free of infection was defined as no *S. aureus*, *P. aeruginosa* or *B. cepacia* in sputum samples (minimum of four samples) over the previous 12 months. Sputum samples were obtained as a part of routine clinical care. Before sampling, all participants rinsed their mouth with water. Each subject provided two single mouth-exhaled breath samples that were collected in separate 3-L Tedlar bags (ProCare B.V., Groningen, the Netherlands) at a constant flow rate of 300 mL·s<sup>-1</sup> using a breath sampler (Loccioni, Angeli di Rosora, Italy) [10]. Briefly, the participants exhaled through a bacterial filter (Air Safety Limited, Morecambe, UK) and a non-rebreathable T-piece (Vacumed, Ventura, CA, USA) which were connected to a CO<sub>2</sub> sensor and a calibrated buffer pipe. From the total exhalation, the dead space air (150 mL) was discarded and the remaining part collected in the sampling bag. The breath samples were collected 90 s apart and stored for a maximum of 8 h prior to analysis. Quantification of HCN in breath was performed using laser-based photoacoustic spectroscopy as described earlier [8, 11]. Exhaled HCN levels were normalised to respiratory CO<sub>2</sub> concentration and mean values were considered for analysis.

To validate whether the particular *S. aureus* strains from randomly selected *S. aureus*-infected patients in whom HCN was detected in breath were capable of producing HCN, we measured the HCN production from these five separately cultured *S. aureus* strains. The strains were isolated from sputum that was collected on the day of breath sampling. The total production of HCN *in vitro* over 30 h was compared with the level in breath of the corresponding patients.

All *S. aureus* clinical isolates *in vitro* produced HCN, although the production level was different across the strains (figure 1a). Production started at approximately 3 h, reached a maximum at 6–7 h, and decreased towards the detection limit 24–30 h after inoculation. The absolute production ranged from (mean $\pm$ sd) 45.8 $\pm$ 37.4 (isolate 3) to 994.2 $\pm$ 807.0 nmol (isolate 2) over 30 h.

The growth of all *S. aureus* strains was monitored for 48 h from the time of inoculation (data not shown). All strains showed comparable concentrations in the stationary phase ( $10^8$ – $10^9$  CFU·mL<sup>-1</sup>). Clinical isolate 1, 2 and 3 reached the stationary phase at 16 h after inoculation, while number 4 and 5 grew slower and reached the stationary phase after approximately 20 h.

We included 17 CF patients infected with *S. aureus* (10 with *S. aureus* in sputum on the day of breath sampling and seven with *S. aureus* in  $>50\%$  of sputum samples, median (interquartile range (IQR)) age 15 (11–31.5)

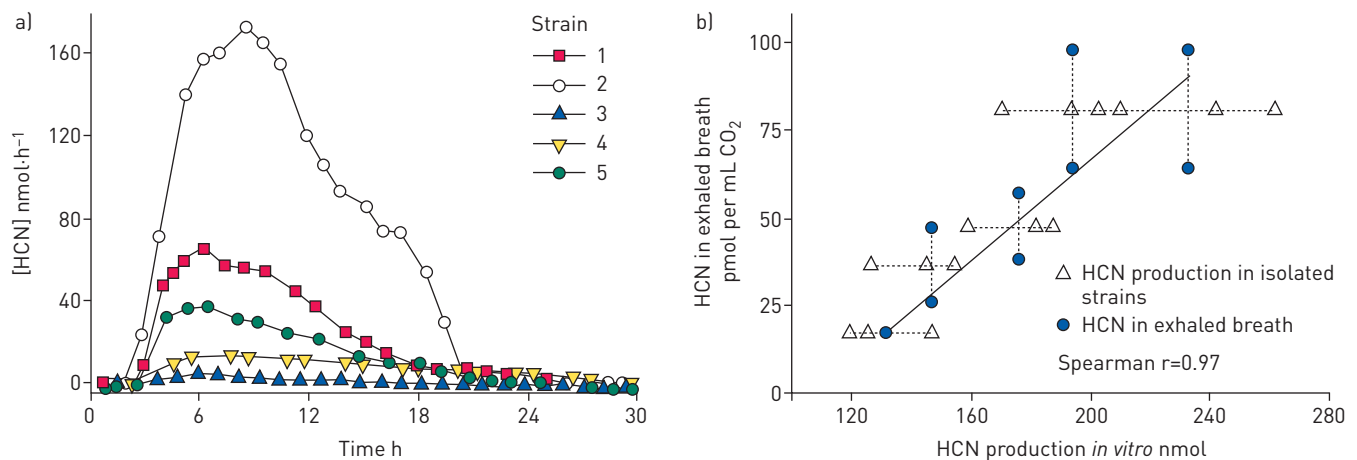


FIGURE 1 Hydrogen cyanide (HCN) production of 10 *Staphylococcus aureus* strains. a) *In vitro* production rates of five clinical *S. aureus* strains from sputum of cystic fibrosis patients. b) HCN production by five *S. aureus* strains isolated from sputum of cystic fibrosis patients [other than those in a) collected on the day of breath sampling in triplicate versus the concentration of HCN in exhaled breath in duplicate.

years), and a combined group of eight CF patients free from *S. aureus* infection and 12 healthy controls (median (IQR) age 24 (21–28) years). Exhaled HCN levels of *S. aureus* infected CF patients, were significantly higher compared to the combined group of non-infected CF patients and healthy controls (median (IQR) 38.0 (25.9–71.3) versus 21.9 (6.8–32.8) pmol·mL<sup>-1</sup> CO<sub>2</sub>, p=0.01). No statistical difference was found for HCN levels in breath of non-infected CF patients compared to healthy controls (median (IQR) 21.7 (7.0–48.6) versus 19.6 (3.4–30.7) pmol·mL<sup>-1</sup> CO<sub>2</sub>, p=0.85).

The HCN production levels of the *S. aureus* strains isolated from sputum on the day of breath sampling were higher compared to breath HCN levels and were strongly correlated (Spearman r=0.97) (figure 1b).

This study demonstrates that HCN is produced by *S. aureus in vitro* and is present in exhaled breath of *S. aureus* infected CF patients. GILCHRIST *et al.* [9] cultured five strains of *S. aureus* on blood agar and reported HCN concentrations of 1–6 parts per billion by volume at 24, 48, 72 and 96 h, which are lower compared to those measured in our study. Variations in strain, culturing method (planktonic versus biofilm conditions), medium (brain-heart infusion versus blood agar) or sampling time-points may account for these differences. By comparing the HCN production dynamics with bacterial growth curves it can be concluded that *S. aureus* starts to produce HCN in the exponential growth phase and varies between strains. HCN is a highly toxic compound, well-known to inhibit cellular respiration [12]. We speculate that the differences in absolute production may contribute to differences in virulence between *S. aureus* strains, since HCN is associated with lung damage, impaired lung function [13] and reduction of the host response effectiveness in CF [11]. Therefore, patients with elevated HCN levels might benefit from aggressive antibiotic treatment in early CF lung disease. The HCN levels in *S. aureus* infected patients are significantly higher compared to a combined group of non-infected patients and healthy controls. The correlated HCN levels *in vitro* and in breath indicate that the breath HCN levels originate from *S. aureus* infection in the lung.

Our results show that HCN in exhaled breath is not an exclusive biomarker for *P. aeruginosa* infection, which is a significant drawback in the attempt to develop a specific diagnostic tool for early detection of *P. aeruginosa* in patients with CF.



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Hydrogen cyanide is produced by *S. aureus in vitro* and *in vivo* and is not an exclusive biomarker for *P. aeruginosa* <http://ow.ly/4nsh7y>

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# Pouched rats as detectors of tuberculosis: comparison to concentrated smear microscopy

*To the Editor:*

In 2014, 1.5 million people died of tuberculosis (TB), a disease that can be cured in nearly every case if detected in time. Rapid and accurate detection of TB is a crucial component of the World Health Organization's 2016–2035 End TB Strategy [1]. Pouched rats, *Cricetomys ansorgei* (previously called *Cricetomys gambianus* [2]), are able to detect *Mycobacterium tuberculosis* by sniffing sputum samples [3]. Since 2007, they have been used for second-line screening of sputum samples previously evaluated by Ziehl–Neelsen microscopy (ZN) at clinics in Tanzania. Use of the rats increases new case detections by ~40% [3].

In 2013, a TB-screening programme using rats opened in Maputo (Mozambique). Here, as in Tanzania, presumptive TB patients provide two samples, which the rats evaluate by sniffing holes above pots containing the heat-inactivated sputum. The rats are taught to pause above samples deemed TB positive by ZN. Samples deemed TB negative by ZN but TB positive by rats are evaluated by concentrated smear light-emitting diode fluorescence microscopy (LED-FM) [4, 5]. Presumptive TB patients with at least one LED-FM-positive sputum sample are assumed to have active TB and constitute additional case detections. Information about the disease status of such patients is conveyed to clinics so that they can be treated. Although sputum concentration and the use of LED-FM (rather than ZN) result in higher sensitivity, TB clinics in Mozambique and other financially impoverished countries are rarely able to implement these methods due to resource constraints [5, 6].