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Variation in hydrogen cyanide production between different strains of *Pseudomonas aeruginosa*

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ABSTRACT: There is increasing interest in using the cyanogenic properties of *Pseudomonas aeruginosa* to develop a nonmicrobiological method for its detection. Prior to this, the variation in cyanide production between different *P. aeruginosa* strains needs to be investigated.

Hydrogen cyanide (HCN) released into the gas phase by 96 genotyped *P. aeruginosa* samples was measured using selected ion flow tube-mass spectrometry after 24, 48, 72 and 96 h of incubation. The HCN produced by a range of non-*P. aeruginosa* cultures and incubated blank plates was also measured.

All *P. aeruginosa* strains produced more HCN than the control samples, which generated extremely low levels. Analysis across all time-points demonstrated that nonmucoid samples produced more HCN than the mucoid samples ($p=0.003$), but this relationship varied according to strain. There were clear differences in the headspace HCN concentration for different strains. Multivariate analysis of headspace HCN for the commonest strains (Liverpool, Midlands_1 and Stoke-on-Trent, UK) revealed a significant effect of strain ($p<0.001$) and a borderline interaction of strain and phenotype ($p=0.051$).

This evidence confirms that all *P. aeruginosa* strains produce HCN but to varying degrees and generates interest in the possible future clinical applications of the cyanogenic properties of *P. aeruginosa*.

KEYWORDS: Microbiology, *Pseudomonas aeruginosa*

The opportunistic organism *Pseudomonas aeruginosa* is of particular significance in cystic fibrosis (CF) due to its association with increased morbidity and mortality [1]. Initial infection usually occurs with a nonmucoid strain that is sensitive to anti-pseudomonal antibiotics. Early, aggressive treatment can therefore result in eradication [2]. As the infection becomes chronic, the *P. aeruginosa* phenotype changes to mucoid, making eradication unlikely [3]. Although most patients are infected by their own individual *P. aeruginosa* strain, siblings often carry the same strain indicating infection from a common environmental strain or from cross-infection [4]. *P. aeruginosa* strains found in more than one patient (clonal strains), which are often multiple-antibiotic resistant and transmissible, have been identified in populations with CF in the UK and Australia [5–7]. This has had a major impact on the infection control precautions necessary for patients with CF and further highlights the need for prompt, accurate *P. aeruginosa* diagnosis.

In children who are unable to expectorate sputum, current methods of *P. aeruginosa* diagnosis are unreliable (cough swab/cough plate) [8–10], unpleasant (induced sputum) [11] or invasive (bronchoscopy) [12]. There is an urgent need for a reliable, noninvasive method of diagnosing *P. aeruginosa* and there is significant interest in whether this could be based on the ability of *P. aeruginosa* to synthesise cyanide [13–15]. *P. aeruginosa* is one of a limited number of organisms to have this property and the only such organism frequently found in the respiratory tract of children with CF [16]. Using a cyanide ion-selective electrode to measure the CN^- ions in aqueous solution, cyanide levels of 300–500 μM have been found in *P. aeruginosa* cultures [16, 17] and of up to 130 μM in the sputum of CF and non-CF bronchiectasis patients with *P. aeruginosa* infection [14, 15].

When hydrogen cyanide (HCN) is in aqueous solution at a neutral pH it is mainly undissociated, meaning it is readily released into the

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headspace. Selected ion flow tube-mass spectrometry (SIFT-MS) has therefore been used to measure gas phase HCN. Using this methodology the HCN concentration has exceeded 17,000 parts-per-billion (ppb) by volume in the headspace (volume 200 mL) over *P. aeruginosa* cultures [18] and 60 ppb in the breath of CF patients infected with *P. aeruginosa* [13]. Conversely, cyanide is essentially absent from the sputum of CF and non-CF bronchiectasis patients without *P. aeruginosa* infection and from the sputum of healthy controls [14, 15]. HCN is also very low or absent in the breath of healthy patients [13, 19]. One of the possible limiting factors in using the cyanogenic property of *P. aeruginosa* in the development of a diagnostic technology is that some cultures do not seem to produce significant levels of cyanide [18, 20]. Similarly, a minority of breath and sputum samples from *P. aeruginosa* positive patients do not have detectable cyanide levels [13–15].

The ability of *P. aeruginosa* to employ quorum sensing and the subsequent relationship between cyanide concentration and bacterial load, supports the hypothesis that the observed variation in cyanide production is dependent on *P. aeruginosa* strain. Quorum sensing is the process by which organisms use extracellular molecules to regulate phenotypes in response to population density. Cyanide production by *P. aeruginosa* is regulated, in part, by quorum sensing, with high population densities inducing synthesis [21–23]. Despite this, cyanide levels have been shown to be independent of *P. aeruginosa* bacterial load when measured in the sputum of CF and non-CF bronchiectasis patients, suggesting cyanide production is dependent on the presence of specific *P. aeruginosa* strains [14]. This hypothesis is also supported by the finding that certain clonal strains of *P. aeruginosa* overproduce specific quorum-sensing regulated exoproducts [24]. This is the first reported study to assess the variation in cyanide production by different *P. aeruginosa* strains.

It is possible that the airway inflammation caused by *P. aeruginosa* infection results in cyanide production by leukocytes. This is supported *in vitro* by reports of leukocytes challenged with *Staphylococcus epidermidis* producing HCN [25, 26] and a recent study from the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) team who found the neutrophil number in bronchoalveolar lavage (BAL) samples to be a predictor of cyanide concentration [27]. However, BAL neutrophil count is significantly higher in patients infected with *P. aeruginosa* compared with those with no infection or infection with other organisms [28] and previous studies have not identified cyanide in patients infected with organisms other than *P. aeruginosa* [13–15]. The high percentage of *P. aeruginosa* negative patients in whom cyanide was identified in the AREST CF study [27] is in contrast to previous papers and this is likely to be the reason why the authors were unable to use cyanide to differentiate between the patients who had *P. aeruginosa* infection and those they believed to be free from *P. aeruginosa*.

METHODS

The HCN concentration in the headspace over *P. aeruginosa* cultures was analysed using SIFT-MS. This technique combines fast-flow tube technology and quantitative mass spectrometry to allow accurate quantification of volatile compounds. Precursor ions, selected according to the compounds to be detected and quantified, are generated in a discharge ion source, mass selected by a quadrupole mass filter, and then injected as selected ionic species into fast-flowing helium carrier gas in a flow tube. The gas/headspace to be analysed is sampled directly into the carrier gas/precursor ion swarm *via* a calibrated capillary. The precursor ions react with the trace gases in the sample producing ions that are characteristic of the trace gas molecules present in the sample. These product ions, and the precursor ions and their hydrates can be detected by a downstream quadruple mass spectrometer. An on-line computer will immediately calculate the partial pressures of the trace gases in the air sample from the ion count rates [29, 30].

As part of a previous study looking at cross-infection between CF patients, all *P. aeruginosa* isolates obtained from CF patients between January and May 2007 had been genotyped [31]. These isolates were stored on Microbank® cryogenic beads (Pro-lab Diagnostics Inc., Neston, UK) at -70°C in our microbiology laboratory (Dept of Microbiology, University Hospital of North Staffordshire, Stoke-on-Trent, UK). For this study, eight new cultures were prepared from each original isolate. This was done by placing a bead from each isolate directly onto a blood agar plate (Oxoid Ltd, Basingstoke, UK) and plating it out. From this plate out, eight blood agar plate cultures were then prepared. Each of these eight plates were covered with a lid, sealed in plastic bags (total volume ~200 mL) and incubated at 37°C. From each original isolate, the headspace over two new plates (referred to later as a paired *P. aeruginosa* cultures) were analysed at 24 h, two at 48 h, two at 72 h and two at 96 h. During analysis the lid was removed from the plate whilst keeping the bag sealed. The plastic bag was pierced with a hypodermic needle attached directly to the input port of the SIFT-MS instrument. The accumulated HCN concentration was analysed for 100 s and the mean concentration over this time was recorded. HCN analysis was performed using the count rate of the product ion H_2CN^+ generated using H_3O^+ precursor ions as previously described [32]. After analysis the bacterial cultures were appropriately discarded.

Using the same methodology five control cultures of *Streptococcus pneumoniae, Staphlococcus aureus, Moraxella catarrhalis, Haemophilus influenzae* and *Aspergillus fumigatus* were each plated onto eight agar plates and analysed after the same periods of incubation. Chocolate agar plates (Oxoid Ltd) were used for the *H. influenzae* samples, Sabouraud agar (Oxoid Ltd) for the *A. fumigatus* samples and blood agar (Oxoid Ltd) for the others. 10 blank blood agar plates and five blank chocolate agar plates were also analysed after the same periods of incubation.

Statistical methods

In order to assess the reliability of the process the intraclass correlation (ICC) was calculated using a two way random effects model. As the raw data was heavily skewed and remained non-normal even after a log transformation, the 95% confidence interval (CI) for the ICC was generated using 1,000 bootstrapped samples. The Shapiro–Wilks' test confirmed that the differences remained non-normal even after transformation of the original values. Consequently median differences between the paired samples at each of the four time-points were generated with corresponding 95% CI. In this way the magnitude of differences between the paired samples at each of the time-points could be assessed.

The HCN concentration for the 96 pairs of *P. aeruginosa* cultures was then averaged at each time-point. A repeated-measures mulitvariate ANOVA (Wilks' lamda) was used to compare the overall distribution of HCN production between phenotypes (mucoid or nonmucoid). A two-way multivariate ANOVA was also adopted to assess the effect of the three commonest strains and phenotype across all time-points. In both cases a log transformation was used to ensure homogeneity of covariance matrices across the groups. Scheffé's *post hoc* test was then performed to identify differences between the mucoid and nonmucoid groups at each time-point and similarly between the three strains. These analyses were repeated on the ranked data as a kind of sensitivity analysis. Due to the non-normality and heterogeneity present, median summaries between the two groups at each time-point were generated.

RESULTS

Background data

Of the 98 genotyped *P. aeruginosa* samples that had been stored as part of the previous study, 96 were available for analysis. 48 samples were mucoid and 48 were nonmucoid. There were 26 different strains, 10 of which were clonal strains. Four of these had been previously described: Liverpool (n=8), Midlands_1 (n=19), Midlands_2 (n=4) and Stoke-on-Trent (n=15). The other six had not been previously descibed: clonal_1 (n=4), clonal_2 (n=5), clonal_3 (n=2), clonal_4 (n=3), clonal_5 (n=3) and clonal_6 (n=2). The remaining 16 genotypes were unique to individual patients; 10 were isolated on more than one occasion (sporadic_1: n=5; sporadic_2: n=3; sporadic_3: n=3; sporadic_4: n=2; sporadic_5: n=2; sporadic_6: n=2; sporadic_7: n=2; sporadic_8: n=2; sporadic_9: n=2; and sporadic_10: n=2) and six on a single occasion (sporadic_11–16).

Reliability of HCN concentration between paired P. aeruginosa cultures

The ICC (95% CI) or the HCN measurement between the 96 pairs of *P. aeruginosa* cultures, was 0.97 (0.96–0.98) at 24 h, 0.97 (0.96–0.98) at 48 h, 0.97 (0.96–0.98) at 72 h and 0.96 (0.95–0.98) at 96 h. The median difference in the HCN concentration between the paired *P. aeruginosa* cultures at each of the four time-points was 3 ppb by volume (95% CI -1–6), 3 ppb by volume (95% CI 1–8), 9 ppb by volume (95% CI -1–27) and 5.5 ppb by volume (95% CI -7–29).

Headspace HCN concentrations for P. aeruginosa and controls cultures

The incubated blank plates and the control cultures of *S. pneumoniae, S. aureus, M. catarrhalis, H. influenzae* and *A. fumigatus* produced extremely low levels of HCN at all time-points (<10 ppb). As expected the HCN was significantly higher in the headspace above the *P. aeruginosa* cultures (table 1).

Headspace HCN concentration for mucoid and nonmucoid phenotypes

The comparison of the headspace HCN concentrations between the mucoid and nonmucoid phenotypes suggested increased production by the nonmucoid samples (table 2). This difference was significant when analysed across all time-points (Wilks' lambda: $F_{4,91}=4.35$, p=0.003) and after 24 h only (p=0.008) when the time-points were analysed individually. The same conclusions were reached using the ranked data although the corresponding p-values were 0.018 and 0.007, respectively. Interestingly when headspace HCN concentrations were split according to strain and phenotype (table 4), some strains had higher HCN for nonmucoid samples (Liverpool and Stoke-on-Trent) and some for mucoid samples (Midlands_1).

Headspace HCN concentration for different P. aeruginosa strains

There are clear differences in the headspace HCN concentrations between different *P. aeruginosa* strains, although there is also variability within strains (table 3). Multivariate analysis of the effect of strain, phenotype and the interaction of both on the headspace HCN concentration above the three commonest strains (Liverpool, Midlands_2 and Stoke-on-Trent) was undertaken (table 4). This results showed a significant effect of strain (Wilks' lambda: $F_{8,66}=5.76$, p<0.001), no effect from phenotype (Wilks' lambda: $F_{4,33}=0.78$, p=0.55) and a borderline interaction of strain and phenotype (Wilks' lambda: $F_{8,66}=2.01$,

TABLE 1 Headspace hydrogen cyanide (HCN) concentrations for *Pseudomonas aeruginosa* (PA) cultures, control cultures and incubated blank plates

Agar	Organism	Samples n	HCN concentration ppbv			
			24 h	48 h	72 h	96 h
BI	PA	96	62 (17–188)	155 (30–1327)	743 (74–2670)	831 (89–2948)
BI	SP	5	2 (2–2)	2 (1–2)	1 (1–2)	2 (1–3)
BI	SA	5	1 (1–4)	4 (3–6)	3 (2–4)	2 (2–3)
BI	MC	5	3 (2–6)	3 (2–5)	1 (1–3)	1 (0–2)
Ch	HI	5	2 (1–3)	2 (2–2)	6 (5–6)	2 (2–3)
Sab	AF	5	1(1–2)	3(2–3)	2(1–2)	1(1–1)
BI	Blank	10	2 (1–2)	2 (1–4.5)	2 (2–2)	2 (2–3)
Ch	Blank	5	3 (3–4)	2 (2–2)	3 (2–3)	2 (1–2)
Sab	Blank	5	1(1–2)	2(2–3)	1(0–2)	3(2–4)

Data are presented as median (interquartile range), unless otherwise stated. ppbv: parts per billion by volume; BI: blood; Ch: chocolate; Sab: Sabouraud; SP: *Streptococcus pneumoniae*; SA: *Staphylococcus aureus*; MC: *Moraxella catarrhalis*; HI: *Haemophilus influenzae*; AF: *Aspergillus fumigatus*.

TABLE 2 Headspace hydrogen cyanide (HCN) concentrations for mucoid and nonmucoid *Pseudomonas aeruginosa* samples

Phenotype	Samples n	HCN concentration ppbv			
		24 h	48 h	72 h	96 h
Mucoid	48	43 (12–136)	127 (35–418)	525 (84–2234)	747 (91–3107)
Nonmucoid	48	99 (22–350)	626 (27–2279)	2196 (36–5528)	1441 (78–6429)

Data are presented as median (interquartile range), unless otherwise stated. ppbv: parts per billion by volume.

p=0.051). The lack of effect of phenotype is unsurprising as the Liverpool and Stoke-on-Trent strains had higher HCN concentrations above nonmucoid samples, whereas the Midlands_1 strain had higher concentrations above mucoid samples. Further analysis revealed the headspace HCN concentration above the Stoke-on-Trent strain to be significantly lower than the other two strains at all four time-points.

DISCUSSION

The present study is the first to investigate the variation in cyanide production between different strains of *P. aeruginosa*. This is important as previous studies have been unable to detect cyanide in a proportion of *P. aeruginosa* samples, increasing the possibility that cyanogensis does vary according to *P. aeruginosa* strain [18, 20]. We have identified HCN in the headspace of all the *P. aeruginosa* samples analysed, reflecting the sensitivity of the SIFT-MS instrument. Despite all the samples producing cyanide, there are clear differences in the quantity produced by different *P. aeruginosa* strains. The low levels of cyanide produced by the control cultures supports previous studies showing *P. aeruginosa* is one of a limited number of organisms to produce cyanide.

TABLE 3 Headspace hydrogen cyanide (HCN) concentrations for different *Pseudomonas aeruginosa* strains

Strain	Samples n	HCN concentration ppbv			
		24 h	48 h	72 h	96 h
Liverpool	8	92 (24–150)	525 (157–1397)	2238 (483–3682)	3093 (2495–4501)
Midlands_1	19	85 (55–158)	164 (90–815)	1451 (380–2634)	748 (550–2338)
Midlands_2	4	24 (14–266)	62 (22–1261)	199 (71–1371)	2186 (222–4471)
Stoke-on-Trent	15	16 (10–24)	24 (14–32)	37 (25–181)	75 (28–109)
Clonal_1	4	499 (153–849)	6570 (4281–8033)	5852 (4858–7699)	8727 (7505–8978)
Clonal_2	5	190 (78–306)	1833 (1020–3175)	3241 (1500–7122)	4336 (803–7239)
Clonal_3	2	48 (33–63)	24 (15–32)	173 (100–245)	54 (35–73)
Clonal_4	3	169 (145–174)	776 (626–1029)	7475 (7126–9128)	7483 (7382–8256)
Clonal_5	3	13 (12–15)	22 (21–29)	39 (26–44)	89 (61–97)
Clonal_6	2	194 (105–283)	1084 (564–1605)	1979 (1025–2933)	3527 (1826–5227)
Sporadic_1	5	21 (18–70)	37 (35–124)	46 (34–1274)	154 (62–2301)
Sporadic_2	3	16 (12–35)	27 (15–27)	167 (87–252)	588 (297–741)
Sporadic_3	3	272 (141–307)	1065 (620–1372)	3754 (2087–3965)	1989 (1558–2063)
Sporadic_4	2	16 (15–17)	74 (57–91)	126 (123–129)	62 (47–76)
Sporadic_5	2	22 (17–26)	15 (9–21)	6 (5–7)	10 (9–10)
Sporadic_6	2	1797 (967–2627)	4874 (2725–7024)	5871 (5311–6431)	6432 (5926–6939)
Sporadic_7	2	6406 (4231–8581)	8299 (7460–9139)	9174 (9012–9336)	7453 (7124–7781)
Sporadic_8	2	979 (853–1105)	5361 (3680–7052)	6965 (4928–9001)	4432 (2499–6364)
Sporadic_9	2	45 (34–56)	117 (90–143)	424 (255–593)	789 (684–895)
Sporadic_10	2	2716 (1389–4043)	1037 (830–1244)	8342 (7819–8864)	15714 (14630–16799)
Sporadic_11#	1	946	1285	3505	3585
Sporadic_12#	1	186	499	7559	12325
Sporadic_13#	1	1002	5715	7621	5771
Sporadic_14#	1	113	1127	16612	11923
Sporadic_15#	1	738	2774	6861	12231
Sporadic_16#	1	7	31	75	34

Data are presented as median (interquartile range), unless otherwise stated. ppbv: parts per billion by volume. #: as n=1, absolute values are given.

TABLE 4 Headspace hydrogen cyanide (HCN) concentrations for mucoid and nonmucoid samples of the Liverpool, Midland_1 and Stoke-on-Trent strains

Strain	Samples n	HCN concentration ppbv			
		24 h	48 h	76 h	92 h
Liverpool					
Mucoid	3	12 (11–70)	161 (156–526)	532 (435–988)	2860 (2131–2971)
Nonmucoid	5	123 (62–216)	949 (159–2740)	3341 (3034–4505)	4028 (3104–5921)
Midlands_1					
Mucoid	14	127 (71–184)	182 (138–368)	1598 (816–2753)	804 (725–2393)
Nonmucoid	5	58 (17–85)	87 (16–1240)	167 (29–1079)	544 (68–2183)
Stoke-on-Trent					
Mucoid	7	11 (8–17)	10 (9–28)	56 (16–186)	39 (28–97)
Nonmucoid	8	22 (15–29)	27 (23–33)	36 (33–104)	77 (48–119)

Data are presented as median (interquartile range), unless otherwise stated. ppbv: parts per billion by volume.

P. aeruginosa synthesises cyanide by the oxidative decarboxylation of glycine using a hydrogen cyanide synthase enzyme, encoded by the *hcnABC* gene locus. This process produces four electrons and four hydrogen atoms per glycine molecule. Temperatures of 34–37°C and microaerobic conditions (O_2 <5%) maximise *P. aeruginosa* cyanogenesis but it is inactivated by both atmospheric oxygen and strict anaerobic conditions [16, 17, 33]. *P. aeruginosa* avoids the toxic effects of cyanide on aerobic respiration by active detoxification mechanisms and synthesis of a respiratory chain terminated by a terminal oxidase insensitive to cyanide [34–37]. *P. aeruginosa* may produce cyanide to exclude other pathogens, thereby allowing it to become the dominant organism. This hypothesis is supported by a study showing cyanide to be the mediating factor in the paralytic killing model of *Caenorhabditis elegans* by *P. aeruginosa* [38]. The high concentrations of cyanide identified in the sputum and breath of CF patients [13–15] may also affect lung cellular function contributing to the lung damage caused by *P. aeruginosa* infection.

Traditionally, the organisms other than *P. aeruginosa* known to be cyanogenic are: *Pseudomonas fluorescens, Pseudomonas aureofaciens, Pseudomonas chlororaphis, Chromobacterium violaceum* and *Rhizobium leguminosarum* [16, 39]. These organisms are not usually isolated from patients with CF. More recently *Burkholderia cepacia* complex have also been shown to produce cyanide under biofilm and colonial growth conditions [40]. These are pathogens in the lungs of patients with CF but as they are usually acquired late in the course of the disease they are uncommon in children. Previously, the proposed cut-off for the HCN detected in the headspace over a culture to confirm *P. aeruginosa* was 100 ppb (sensitivity 68% and specificity 100%) [18]. These data suggest that for the cultures included in this study, using the highest HCN concentration at any of the time-point, the cut-off could be reduced to 10 ppb (sensitivity 100% and specificity 100%).

The excellent correlation between the HCN produced by the pairs of *P. aeruginosa* cultures confirms the reproducibility of cyanide production by *P. aeruginosa* isolates and of the SIFT-MS real-time analyses. Although there is variation in the HCN production by samples of the same strain, overall there are clear differences between *P. aeruginosa* strains. This is the first study to show this difference. We have also shown that all the *P. aeruginosa* samples produced detectable levels of HCN, which reflects the high sensitivity and reliability of the SIFT-MS analyses. It is unclear how *in vitro* cyanide production by *P. aeruginosa* cultures correlates with *in vivo* cyanide detection in patient's breath or sputum. Specifically, as *in vivo* cyanide levels tend to be lower [13–15], it is not known if current methods are sensitive enough to detect cyanide in sputum or exhaled breath produced by a *P. aeruginosa* strain with lower cyanide production. It is also possible that the same strain of *P. aeruginosa* could produce different amounts of HCN depending on its position in the respiratory tract and the specific environmental conditions present.

Regarding phenotype, previous studies have reported higher cyanide production by mucoid *P. aeruginosa* cultures [20, 27]. Our data suggest the opposite, with nonmucoid cultures having higher HCN concentrations. This may be related to the timing of analysis, as the trend for HCN concentration was still increasing at 96 h for the mucoid samples, whereas the nonmucoid samples peaked at 72 h and started to fall at 96 h. We may therefore have observed a different effect of phenotype if HCN analysis was undertaken after a longer period of incubation. Interestingly, when the HCN concentrations were analysed for strain and phenotype (table 4), the effect of phenotype seemed to differ between strains. The observed difference in the effect of phenotype on cyanogenesis between this and other studies may therefore be explained by which *P. aeruginosa* strain was included in the various studies.

In conclusion, the present study has demonstrated that all of our *P. aeruginosa* samples produced cyanide, but that the relative levels varied according to strain. This will create interest in the possible future clinical applications of the cyanogenic property of *P. aeruginosa*.

STATEMENT OF INTEREST

None declared.

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