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Original Article

An invisible threat? *Aspergillus* positive cultures and co-infecting bacteria in airway samples

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ABSTRACT

Background: *Aspergillus fumigatus* (Af) infection is associated with poor lung health in chronic suppurative lung diseases but often goes undetected. We hypothesised that inhibition of Af growth by *Pseudomonas aeruginosa* (Pa) increases the frequency of false-negative Af culture in co-infected people. Using a substantial group of cystic fibrosis (CF) airway samples, we assessed the relationship between Af and bacterial pathogens, additionally comparing fungal culture with next-generation sequencing.

Methods: Frequency of co-culture was assessed for 44,554 sputum/BAL cultures, from 1,367 CF patients between the years 2010–2020. In a subgroup, Internal Transcribed Spacer-2 (ITS2) fungal sequencing was used to determine sequencing-positive, culture-negative (S+/C-) rates.

Results: Pa+ samples were nearly 40% less likely ($P<0.0001$) than Pa- samples to culture Af, an effect that was also seen with some other Gram-negative isolates. This impact varied with Pa density and appeared to be moderated by *Staphylococcus aureus* co-infection. Sequencing identified Af-S+/C- for 40.1% of tested sputa. Samples with Pa had higher rates of Af-S+/C- (49.3%) than those without (35.7%; RR 1.38 [1.02–1.93], $P<0.05$). Af-S+/C- rate was not changed by other common bacterial infections. Pa did not affect the S+/C- rates of *Candida, Exophiala* or *Scedosporium*.

Conclusions: Pa/ Af co-positive cultures are less common than expected in CF. Our findings suggest an Af-positive culture is less likely in the presence of Pa. Interpretation of negative cultures should be cautious, particularly in Pa-positive samples, and a companion molecular diagnostic could be useful. Further work investigating mechanisms, alternative detection techniques and other chronic suppurative lung diseases is needed.

1. Introduction

Aspergillus fumigatus (Af), is the commonest fungus in the cystic fibrosis (CF) airway [1] and is linked with worsened structural airways disease, lung function, exacerbation frequency and quality-of-life [2–6]. Azole treatment of Af-colonisation, resulting in significant Af airway biomass reduction, improved CT scores and exacerbation rates [7]. Although prolonged incubation may improve culture detection, standard techniques are suboptimal [8]. This is compounded by the poor accuracy of cough/throat swabs in non-sputum producers, particularly for fungal culture [9,10]. Estimates of Af prevalence in CF range widely (9–74%) depending on detection methodology, are rising and increase with age [3,11–13]. Molecular methods improve detection by >90% compared to culture [14,15], but rely on sputum which is now expectorated rarely by many patients on CFTR modulators.

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Pseudomonas aeruginosa (Pa) is the commonest bacterial infection in CF; chronic infection is strongly linked to morbidity and mortality [16]. Clinical data on Pa/Af co-infection are unclear, but studies have reported detrimental impact on lung function and need for intravenous (IV) antibiotics compared to either pathogen alone [2,5,6]. *In vitro* studies demonstrate that bacterial CF pathogens demonstrate complex interactions with *Aspergillus* [17]. Deeper understanding of the balance of fungi within healthy and diseased airways has come from the use of next generation sequencing approaches to delineate the mycobiome, and to explore the complexities of microbial interactions [18,19].

Pa inhibits Af growth *in vitro,* so we hypothesised this could lead to false-negative Af culture in Pa-infected samples. Our first aim was to assess the frequency of culture co-positivity of bacterial and fungal organisms, with particular focus on Pa and Af. Our second aim was to explore the relationship of Pa with the Af detection gap between culture and molecular, culture-independent methods.

2. Methods

2.1. Study subjects and design

In a longitudinal, single-centre (Royal Brompton Hospital) cohort, all results from sputum and broncho-alveolar lavage (BAL) samples from adults and children with CF (2010–2020) were identified. All samples underwent standard clinical laboratory culture according to CF Trust guidelines (supplement) [20]. Cough/throat swab results were excluded. All patients had consented to data entry into the national CF patient registry whose approval was granted for this study.

As part of a previous study, Internal Transcribed Spacer-2 (ITS2) sequencing had been performed on a subset of sputa [19]. The first of paired samples was sent for routine microbiological culture, and the second was split into 300μl sputum aliquots prior to DNA extraction (supplement). All sequences are available from the European Nucleotide Database (PRJEB33434). The study was approved by Royal Brompton and Harefield Hospital Biomedical Research Unit Ethics Committee (Advanced lung disease biobank 10/H0504/9).

2.2. Statistical analysis

The hypothesis was that Af culture-positivity would be affected by the presence of co-infecting bacteria. Frequency of Af culture-positivity in samples *free* of bacteria was compared with observed frequencies of Af culture-positivity in samples infected with specific bacterial pathogens. This was performed for *all* samples containing each bacterium and, for greater specificity, samples infected with *only* that bacterium. Sensitivity analyses were also performed, first using a single sample from 3-monthly time-periods from each patient, and second by randomly selecting 10 samples from each patient who had at least 10 culture results recorded in order to reduce bias from patients with high sample numbers. Age at sampling midpoint was used for patient-based analysis. We planned to compare observed with expected frequencies of co-culture (where expected frequency = observed frequency of pathogen A x observed frequency of pathogen B / 100), using Pearson's chi-squared test to examine for difference. The Mantel-Haenzsel test was applied for trends across a contingency table, reporting a '*P* for trend' (p*trend*) statistic. Contingency tables examined the performance of fungal culture versus ITS2 fungal sequencing in the presence or absence of co-infecting bacteria, using relative risk (RR [95% CI]) and Chi-square testing. SPSS Statistics (version 27; IBM, Armonk, NY) was used for data processing and statistics. Data are median (IQR) unless stated. $P<0.05$ was considered significant after corrections for multiple comparisons.

3. Results

3.1. Prevalence of bacterial and Aspergillus positive samples

44,554 sputum/ BAL results were available: 1367 CF patients with median (IQR) age 24.1 (13.6–35.5) years contributed 19 (5–51) samples each over 6.0 (2.2–9.6) years. 4844 (10.9%) were completely negative cultures (no bacterial or fungal growth), 39,710 were positive for at least one bacteria or fungus. For individual bacteria: Pa in 24,833 (55.7%), Sa in 11,163 (25.1%), *Burkholderia cepacia* complex (Bcc) in 1874 (4.2%), *Achromobacter xylosoxidans* (axe) in 1880 (4.2%) and *Stenotrophomonas maltophilia* (Sm) in 1635 (3.7%). Af was grown from 5189 (11.6%) samples.

3.2. Determining the relationship between bacterial culture positivity and Af growth

We hypothesised that bacterial co-infection would influence the frequency of Af growth. Across the whole sample set, we first assessed frequency of Af culture-positivity in bacteria-negative samples: 1525/9818 (15.5%) (Table 1). We then compared this with the frequency of Af in samples positive for bacteria: for Pa, Af was detected significantly less frequently (9.8%; RR 0.63 [0.60–0.66], $P<0.0001$) (Fig. 1). Similar was seen for Sa (RR 0.82 [0.77–0.88], $P<0.0001$), Bcc (0.35 [0.28–0.44], $P<0.0001$) and axe (0.35 [0.28–0.43], $P<0.0001$). In contrast, Af occurred *more* frequently than expected in samples co-infected with Sm (RR 1.20 [1.03–1.39], $P<0.001$).

3.3. Identifying specific relationships with individual bacterial pathogens

We analysed the 16,298 (36.6%) samples in which only a *single* bacterial species (+/- fungus) had grown. The relationships above were again observed, with the exception of Sa (Tables 1, S1 and Fig. 1).

3.4. Sensitivity analyses to reduce confounding from subjects with frequent samples

In order to minimise the potential impact of sampling frequency from certain subjects, we performed a number of sensitivity analyses, each of which supported the previous findings. The first included only one sample per subject for each quarter year: 19,987 samples, 1367 patients, 10 (3–25) samples/ subject over 6.0 (2.1–9.6) years; median sampling interval was 0.28 (0.22–0.41) years. We started by assessing Af culture-positivity in bacteria-negative samples (10.3%). Af was again less common with Pa (9.5%; RR 0.92 [0.85–0.99], $P<0.01$). The previously described relationships with the other bacterial species persisted (Table S2), except for Sa which appeared positively associated with Af (12.3%; RR 1.19 [1.08–1.31], $P<0.0001$).

A second analysis was performed by randomly selecting 10 samples from each patient who had at least 10 culture results recorded. Of these 8650 samples, 13% (1126/8650) had no bacterial/fungal growth. 4526 (52.3%) grew Pa, 933 (10.8%) grew Af. Here, the expected Pa/Af co-positivity was 5.65% ($(52.3 \times 10.8)/100$), whereas the observed frequency was 4.65% (RR 0.83 [0.72–0.94], $P<0.01$).

3.5. Subgroup of patients with Af and either Pa or Sa infection: assessing frequency of co-infected samples

Pa: 494 patients had samples positive for Pa and samples positive for Af during the study period. Assessing all their 26,548 samples, 15,743 (59.3%) grew Pa and 4752 (17.9%) grew Af. We rea-

Table 1
Analysis of all 44,554 samples. Expected (15.5%) vs actual frequency of Af isolation expressed as relative risk (95% CI) and grouped by bacterial co-infection. This analysis is performed for all samples (n = 44,554) and for samples containing a single bacterial species (n = 16,298). *** = P<0.001, **** = P<0.0001, ns = not significant.

	n (%)	Expected Af freq (15.5%), n (%, 95% CI)	Actual Af freq, n (%, 95% CI))	*All samples* Relative risk (95% CI), Actual/Expected	*Single bacteria* Relative risk (95% CI), Actual/Expected
No bacteria	9818	–	1525 (15.5, 14.8–16.2)	–	–
P. aeruginosa	24,833 (55.7)	3849 (15.5, 15.0–16.0)	2423 (9.8, 9.4–10.2)	0.63 (0.60–0.66) ****	0.63 (0.59–0.67) ****
S. aureus	11,163 (25.1)	1730 (15.5, 14.8–16.2)	1427 (12.8, 12.2–13.4)	0.82 (0.77–0.88) ****	0.94 (0.84–1.05), ns
B. cepacia complex	1874 (4.2)	290 (15.5, 13.5–17.1)	102 (5.4, 4.4–6.4)	0.35 (0.28–0.44) ****	0.38 (0.27–0.53) ****
A. xylosoxidans	1880 (4.2)	291 (15.5, 13.5–17.1)	101 (5.4, 4.4–6.4)	0.35 (0.28–0.43) ****	0.41 (0.30–0.58) ****
S. maltophilia	1635 (3.7)	253 (15.5, 13.7–17.3)	303 (18.5, 16.6–20.4)	1.20 (1.03–1.39) ***	1.73 (1.34–2.22) ****

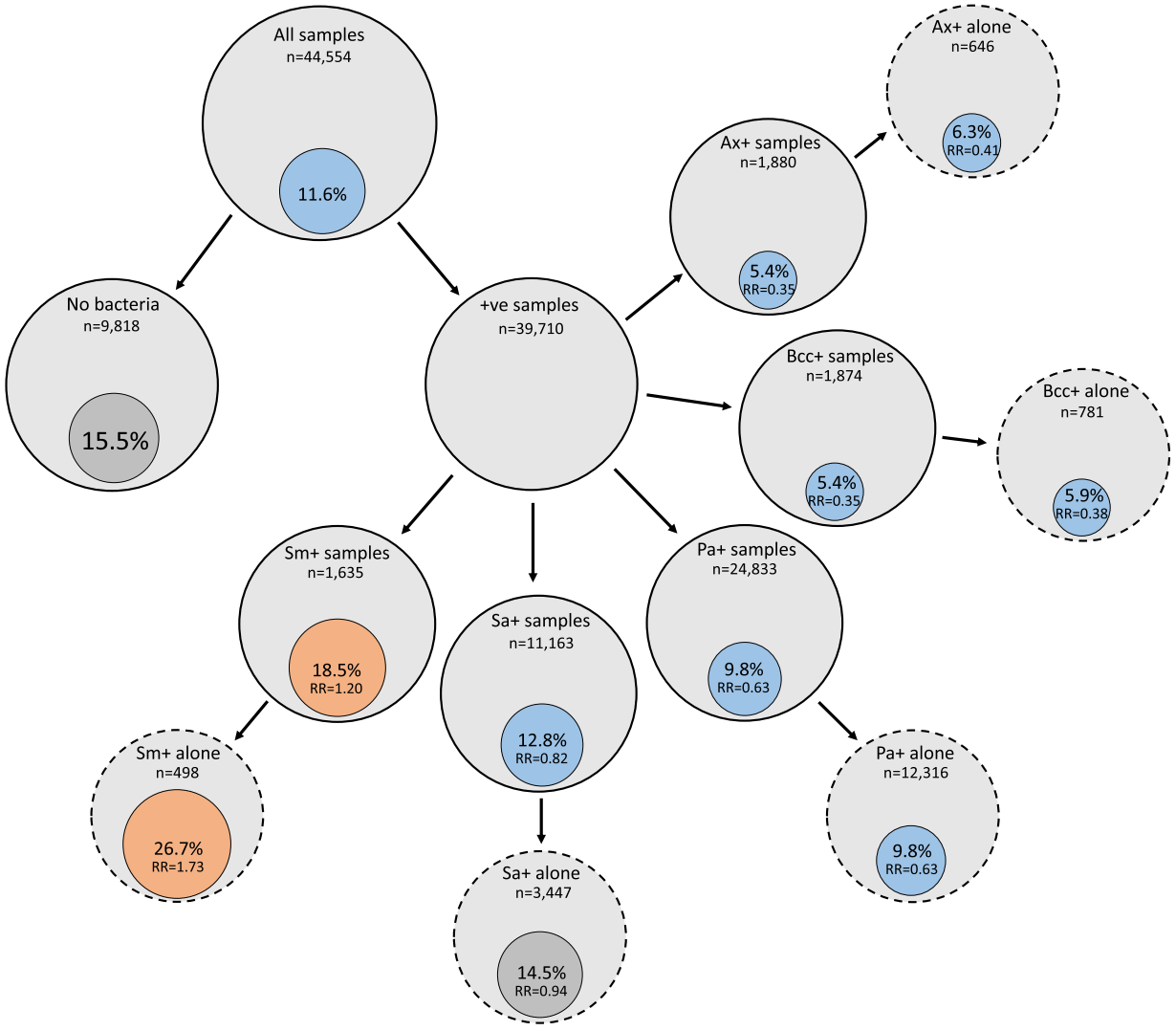

Fig. 1. Schematic representation of selected bacterial and Af culture associations, including relative risk (RR) of Af growth compared to benchmark (no bacteria group; 15.5%). Large grey circles = sample group; solid line for all samples, dashed for single bacterial infection samples. Inner circles = Af positive sub-group; grey = no change in Af frequency compared to benchmark, blue = significant decrease in Af frequency (RR<1), orange = significant increase in Af frequency (RR>1).

soned that in the absence of any interaction, *expected* Pa/Af co-positivity would be the product of these frequencies, ie. 10.6% ((59.3 × 17.9)/100). *Observed* frequency was 9.1% (RR 0.86 [0.82–0.91], P<0.0001).

Sa: 422 patients had at least one growth of Sa and Af and contributed 22,524 samples: 32.2% Sa and 19.1% Af. Expected (6.1%) versus observed (6.3%) co-positivity frequencies were not different (RR 1.03 [0.96–1.11], ns).

Due to low subject numbers, this analysis was not performed for other bacterial pathogens.

3.6. *The relationship between* bacterial density *and Af co-infection*

If Pa infection was associated with a lower chance of Af culture positivity, we reasoned this should be more apparent with heavy Pa infection. Across the whole sample set, of those samples in which the only bacterium cultured was Pa (12,269), Af was grown from 98/646 (15.2%, 12.4–18.0) of those with the lowest Pa density (10^3 cfu/ml) and 400/5167 (7.7%, 7.0–8.4) with the highest density ($\geq 10^7$ cfu/ml, p*trend* <0.0001) (Fig. 2, Table S3). For samples in which the only bacterium cultured was Sa, a trend in the

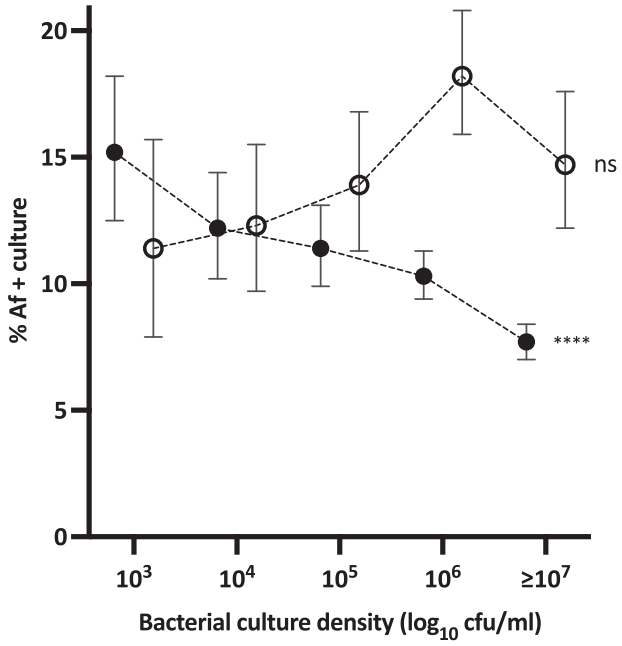

Fig. 2. Percentage (+/- 95% CI) of samples culture positive for Af against log_{10} cfu/ml bacterial density when Pa or Sa are the only bacterial culture growth. *P. aeruginosa* = filled circles, *S. aureus* = clear circles. **** = p*trend* <0.0001, ns = p*trend* not significant.

opposite direction was seen, although this did not reach statistical significance: Af cultured from 31/273 (11.4%, 7.6–15.2) of samples with lowest density Sa (10^3 cfu/ml) and 104/706 (14.7%, 12.1–17.3) with highest density (≥10^7 cfu/ml).

3.7. Exploring the complex interactions between Pa, Sa and Af

We were observing a clear signal from Pa, with presence of the bacterium associated with lower than expected culture positivity of Af, and more exaggerated findings in densely infected samples. The signal from Sa was less clear, differing at various stages of this analysis. As Pa/Sa co-infection is common, we sought to interrogate this further in Pa/Sa co-infected samples (a) and subjects (b).

(a) ***Af in dual Pa/ Sa co-infected samples***

Of all samples, 2653/44,554 (6.0%) were Pa/Sa co-infected; 352 (13.3%) of them also cultured Af. Culture density was used to categorise light (10^3–10^5 cfu/ml) and heavy (10^6–10^8 cfu/ml) growths for each bacterial species. Af culture-positivity was significantly less common in association with heavy Pa and light Sa growths (9.2%) compared to light Pa and heavy Sa (19.1%. P<0.0001). There were significantly fewer Af-positive cultures in the presence of heavy vs light Pa, both when Sa was light (9.2% vs 14.2%, P<0.05) and heavy (12.9% vs 19.1%, P<0.05).

(a) ***Af growth in samples from patients with both Pa and Sa during the study period***

In contrast to limiting analysis to only dual bacterially-infected samples as above, we here included all samples from the 381 (27.9%) patients that had grown Pa, Sa and Af at any time, either alone or together, during the study. These patients contributed 21,136 samples: 56.6% (55.9–57.3) grew Pa, 31.5% (30.9–32.1) grew Sa and 18.9% (18.4–19.4) grew Af. Af was more commonly grown from samples with Sa not Pa (22.8%), than in those with Pa not Sa (15.9%. P<0.0001). Of the 3421 samples co-positive for Pa and Sa, 17.2% (15.9–18.5) also grew Af. The frequency of Af culture-positivity varied within these samples depending on Pa/Sa density in a manner similar to that seen in (a) (Fig. 3, Table S4). With highest Pa density (10^7 cfu/ml) and lowest Sa density (10^3 cfu/ml), Af was grown in 8/66 (12.1%, 4.2–20) samples. Conversely, with lowest Pa density and highest Sa density, Af was grown in 21/56 (37.5%, 24.8–52) samples (p*trend* <0.0001). At high Sa density (10^7 cfu/ml), Af frequency decreased as Pa density increased (p*trend* <0.001). Conversely, at high Pa density, Af frequency increased as Sa density increased (p*trend* <0.05).

3.8. Culture vs non-culture based analysis

Having observed a lower-than-expected frequency of Af co-infection in samples positive for Pa and demonstrated this is greatest at highest Pa densities, confirmed in both patients known to harbour both pathogens and Pa/Sa dual-infected samples directly, we hypothesised that Pa was inhibiting Af present in a sample from growing in culture. ITS2 fungal sequencing data were available for 329 sputa from 124 patients; median age 29.5 (23.0–37.0) years, 2 (1–4) samples per patient (fungal community composition outlined in Fig. S1 of supplement). Two hundred and 34 (71.1%, 66–76) samples were Pa culture-positive and 38 (11.6%, 8.1–15.1) Af culture-positive. ITS2 sequencing identified Af in 160 (48.6%, 43.2–54.0) samples; 132 (82.5%, 76.6–88.4) of these were Af culture-negative, implying that 132/329 (40.1%, 35.8–46.4) were 'sequencing-positive, culture-negative' (S+/C-) (Fig. 4). These Af-S+/C- samples arose from 84 patients, 55% of whom had at least one Af positive culture at another point during the study period. We explored whether Af-S+/C- rate was related to presence of co-infecting bacteria: Af-S+/C- was significantly more common in the presence of Pa (102/234 [43.6%, 37.3–49.9] vs Pa-neg 30/95 [31.6%, 22.2–40.9]; RR 1.38 [1.01–1.94], P<0.05) but was not impacted by co-infection with Sa, Sm or axe. Neither Pa nor Sa culture positivity was associated with an increased rate of S+/C- samples for *Candida* or the non-*Aspergillus* filamentous fungi *Exophiala* or *Scedosporium*. Importantly, we found no significant association between the use of anti-fungal medication and Af-S+/C- rate in patients' first analysed samples (n = 124, 24/77 [31.2%, 20.9–41.5] with anti-fungal vs 11/47 [23.4%, 11.3–35.5] without anti-fungal, ns).

4. Discussion

In this large study, we describe a significantly reduced frequency of *Aspergillus* culture-positivity in samples infected with *Pseudomonas aeruginosa* and several other Gram-negative respiratory pathogens. The discrepancy between culture and molecular detection leads us to hypothesise that this relates to inter-species competition: the presence of Pa impairs Af growth either under laboratory culture conditions or, as ITS2 testing does not distinguish live from dead organisms, within the airway itself.

Positive Af cultures were seen nearly 40% less often in the presence of Pa than when no bacterial pathogen was isolated, an effect more pronounced with increasing Pa density. This negative association between Pa and Af, in combination with a corresponding increase in sequencing-positive, culture-negative samples, suggests that Pa-free conditions may be favourable for fungal culture either within pockets in the CF airways or on the culture plate. This finding was corroborated by analysis of patients with serial cultures demonstrating both Pa and Af infections, in whom dual-positive cultures were less common than expected mathematically. The relative reduction in Af risk with Pa in sensitivity analyses predominantly reflects the lower Af prevalence in bacteria-negative samples from this smaller group. Future work should elucidate this further by using molecular methods of bacterial/fungal detection on clinical samples and *in vitro* co-infection models.

An even more pronounced negative association with Af was seen with *Burkholderia* and *Achromobacter* isolates, although depth of analysis was limited by lower sample numbers. Bcc has been

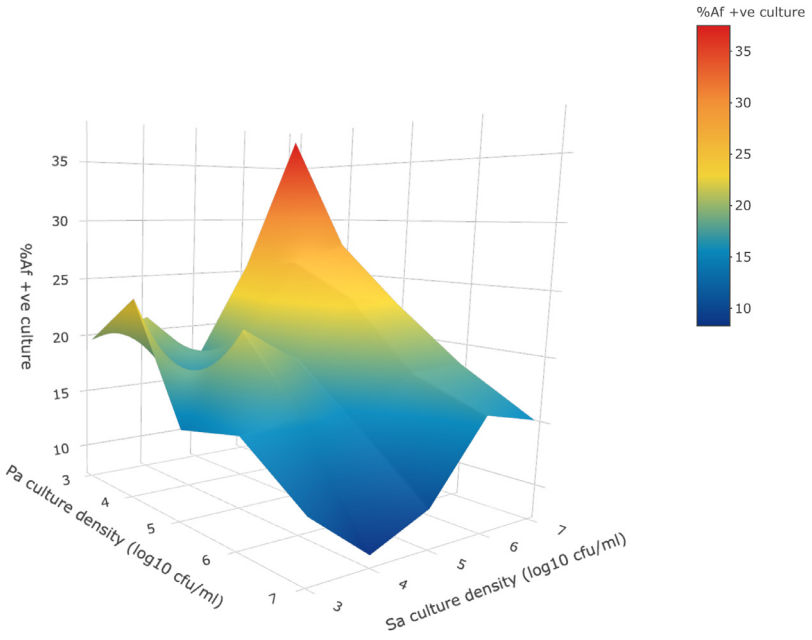

Fig. 3. Heat map illustrating the impact of Pa and Sa culture density on Af culture frequency. Highest frequency of Af culture (red) seen with lowest Pa density (cfu/ml) and highest Sa density. Lowest frequency of Af culture (blue) seen with highest Pa density and lowest Sa density. At any given Sa density, there is a trend towards increasing Af frequency as Pa density decreases. At any given Pa density, there is a trend towards increasing Af frequency as Sa density increases.

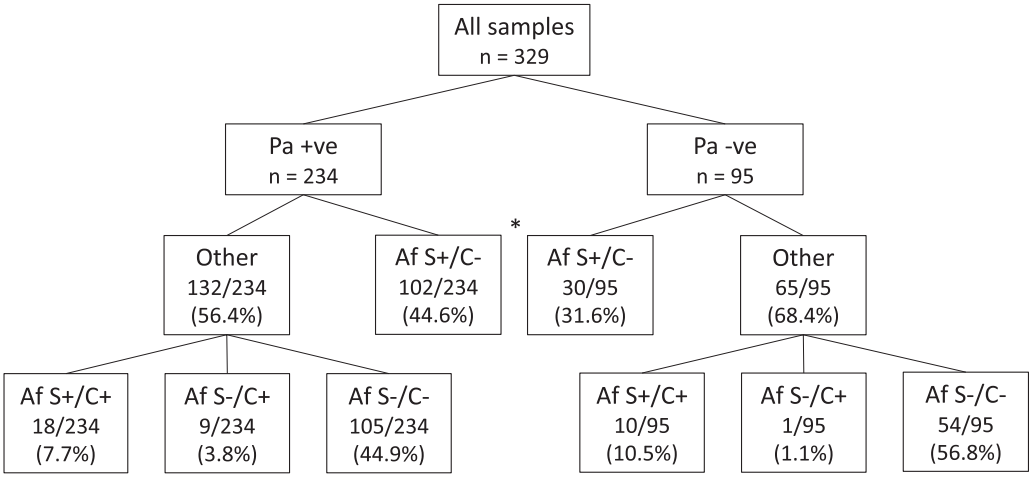

Fig. 4. Flow chart demonstrating the culture and non-culture based analysis of 329 sputum samples, separated depending on *Pseudomonas aeruginosa* (Pa) culture status. Results represented as n (%) (95% CI found in manuscript text). * = $P<0.05$.

demonstrated to inhibit Af *in vitro*, though understanding of the mechanisms behind these interactions remains limited [21]. In contrast, Af was cultured in samples positive for *Stenotrophomonas* significantly more frequently than would be expected. Unfortunately, these bacterial species were not seen with sufficient frequency in the ITS2-sequenced samples to draw conclusions about potential mechanisms. It is biologically plausible that the *Stenotrophomonas*-association reflects a supportive microbiological interaction between these pathogens *in vitro* and *in vivo*, such as has been reported between Pa and Sm [22]. Another study demonstrated that specific co-infection reduced the susceptibility of organisms to antibiotic-mediated killing [23]; it is plausible that species interactions could similarly protect against host defence mechanisms. To our knowledge this has not been studied and is a potential area for future research.

The second most frequent bacterial species, *S. aureus*, led to conflicting findings in different subgroups of analysis. A small apparent reduced risk of Af in Sa-positive samples was lost in samples with Sa as the *only* bacterial infection and was likely confounded by the high rates of Sa/Pa co-infection. Indeed, there was a trend towards increasing frequency of Af growth as Sa culture-density increased which, in the absence of an impact on Af-S+/C- rates, hints at the possibility of an *in vivo* survival advantage of co-infection with Sa. Exploring this further with samples co-infected with Pa/Sa provided an interesting insight into potential interactions with Af. As Pa density increased, Af frequency decreased, but this association appeared to be modulated by Sa. In patients with a history of all 3 pathogens, the rate of Af was higher when samples cultured Sa not Pa, than in those with Pa not Sa. Pa and Sa can adopt complex, often mutually antagonistic, relationships *in vitro*, which are summarised in a recent review [24]. It is not yet clear how this relationship and its impact on immune/inflammatory responses affects CF airways, or behaviour towards other pathogens such as *Aspergillus*.

Fungal sequencing demonstrated an overall Af-S+/C- rate of 40.1%, in-line with other data from CF sputum where 43% of samples were *Aspergillus* culture-negative but PCR-positive [15]. Our novel finding is the significantly increased risk (RR 1.38) of Af failing to grow in samples infected with Pa. This was specific to this fungus/bacterium pair and was not seen with Pa and other fungi. *In vitro* data suggests that Pa and Af have a mutually co-inhibitory relationship, with the effect of Pa appearing dominant, best described as indirectly via the Pa siderophore, pyoverdine [25,26]; there may also be other mechanisms for example contact-dependant killing [27]. Such mechanisms could be active either *in vivo* or *in vitro* following expectoration. Future work should include detailed exploration of these complex inter-kingdom interactions *in vitro*, but should also allow for consideration of intra-kingdom interactions within the whole microbial community using novel approaches such as metagenomics, metatranscriptomics and integrative microbiomics [18].

Our dataset included all positive and negative cultures during the period of interest, giving an overall Af-positive sample prevalence of 11.6%. This is not directly comparable to prevalence within a CF population, where existing estimates appear to be increasing [12]. Higher Af prevalence estimates from CF sputa originate from molecular techniques, reported to increase prevalence from 8 to 35.3% (culture) to 47.9–68% (PCR) [11,14]. Reece *at al.* found a 94% increased detection rate of Af in CF sputum using qPCR compared to standard fungal culture;[14] other studies demonstrated the utility of qPCR for early detection of fungus following azole treatment [7]. Several adjustments to standard fungal culture have narrowed the detection gap with molecular methods [8,14] though this may primarily improve culture sensitivity of non-*Aspergillus* filamentous fungi such as *Scedosporium* [28]. We do not have corroborative PCR data from our samples, but the ITS2 methodology is recognised as sensitive and robust [29]. Alternative detection methods include the measurement of galactomannan, a polysaccharide produced during the logarithmic growth-phase of filamentous fungi. This biomarker is detectable in homogenised CF sputum, improves detection of fungus versus culture alone [13], and could be incorporated into future research protocols exploring pathogen-pathogen interactions. Access to sputum samples in the numbers seen here is likely to decrease in future due to the impact of HEMT. We may see a move towards more widespread use of induced sputum sampling techniques, and it is encouraging that non-sputum based detection methods [30] are being explored. In addition, early data supports reduced airway pathogen culture positivity (bacterial and fungal) in patients on HEMT, thought likely secondary to improved mucociliary clearance and host immune response [31].

The exclusion of cough/throat swab data from analysis means that the group was largely sputum producing and older (median 24.1 years), reflecting a cohort with more advanced lung disease, higher rates of both *Pseudomonas* [32] and *Aspergillus* [1] infection, and perhaps bacterial adaptations. We considered this approach would more accurately represent lower airway infection [9], whilst avoiding a sampling bias against fungal infections;[14] our findings may therefore not extrapolate to young children.

Limitations of the present study include the low prevalence of non-*Aspergillus* filamentous fungi for comparison with the described Af-bacterial associations. The sequencing data however does not suggest that large numbers of these infections are being missed on culture. Another limitation is a lack of contemporaneous clinical information such as anti-microbial therapies. Oral anti-fungal medication use in the adult CF population can be high and may in part explain declining Af prevalence during older age. Our previous UK registry study suggested that anti-fungal prescribing is increased in those with chronic Pa irrespective of their Af status, presumably based on clinical markers of disease severity [5]. We might expect that anti-fungal therapy would increase the proportion of S+/C- samples, though we did not see evidence of this in the small cohort of patients from whom we had sputum ITS2 sequencing data. Nebulisation of the polymyxin-antibiotic colistin is widespread for Pa treatment in Europe. Colistin has been reported to have some *in vitro* anti-fungal activity [33], though a recent study only reported this in combination with isavuconazole against non-*fumigatus Aspergillus* strains [34]. Whilst it seems plausible that the presence of anti-fungal compound in the sputum of a chronic Pa patient may impact on these results, our within-patient analyses, in particular the significant dose relationships with Pa density, would argue against this and suggest a mechanism related to the bacterium itself. Other studies have reported an increase in Af prevalence with nebulised Pa eradication treatment [35] and chronic suppressive therapy [36–39] which would be complimentary to this data, suggesting that a reduction in Pa prevalence or density may lead to an increase in Af recovery on culture.

Failure to detect Af infection has significant implications for the treatment, health and well-being of people with CF, and likely also for others with chronic suppurative lung diseases. For the first time, we report the strong relationship of Af culture-negativity with specific bacterial infections, in particular Pa. We recommend caution in the interpretation of fungal culture data alone, particularly negative fungal cultures in Pa-infected samples. There is an urgent need to develop molecular techniques and/or biomarkers of fungal infection to support decision-making, and to assess the clinical outcomes.

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Author contributions

DAH & JCD designed the study. DAH, NR, LC, IF, NJS, MRL, MFM & WOC contributed to data collection. IF, NJS, MRL, MFM & WOC were responsible for patient recruitment and sequencing sample collection. DAH, MR, NR, LC, HP, IF, DAJ, SJE & JCD contributed to data management and analysis. DAH, MR & HP performed the statistical analysis. DAH, MR & JCD wrote the first draft of the manuscript that was revised and approved by all authors for important intellectual content. All authors approved the final version of the manuscript.

Declaration of Competing Interest

No authors declare competing interests directly relating to this manuscript. NS reports personal fees from Vertex, Chiesi, Gilead, Menarini, Pulmocide, Zambon and Roche. ML reports personal fees from Insmed, Astra Zeneca, and Grifols. JCD reports advisory roles with Algipharma AS, Bayer AG, Boehringer Ingelheim Pharma GmbH & Co. KG, Galapagos NV, ImevaX GmbH, Nivalis Therapeutics Inc., ProQR Therapeutics III B.V., Proteostasis Therapeutics Inc., Raptor Pharmaceuticals Inc., Vertex Pharmaceuticals (Europe) Limited, Enterprise, Novartis, Pulmocide, Flatley, and Teva.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2022.07.009.

References

[1] Pihet M, Carrere J, Cimon B, Chabasse D, Delhaes L, Symoens F, et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis–a review. Med Mycol 2009;47(4):387–97.
[2] Amin R, Dupuis A, Aaron SD, Ratjen F. The effect of chronic infection with *Aspergillus* fumigatus on lung function and hospitalization in patients with cystic fibrosis. Chest 2010;137(1):171–6.
[3] Breuer O, Schultz A, Garratt LW, Turkovic L, Rosenow T, Murray CP, et al. *Aspergillus* infections and progression of structural lung disease in children with cystic fibrosis. Am J Respir Crit Care Med 2020;201(6):688–96.
[4] Hong G, Alby K, Ng SCW, Fleck V, Kubrak C, Rubenstein RC, et al. The presence of *Aspergillus* fumigatus is associated with worse respiratory quality of life in cystic fibrosis. J Cyst Fibros 2020;19(1):125–30.
[5] Hughes DA, Archangelidi O, Coates M, Armstrong-James D, Elborn SJ, Carr SB, et al. Clinical characteristics of *Pseudomonas* and *Aspergillus* co-infected cystic fibrosis patients: a UK registry study. J Cyst Fibros 2022;21(1):129–35.
[6] Reece E, Segurado R, Jackson A, McClean S, Renwick J, Greally P. Co-colonisation with *Aspergillus* fumigatus and *Pseudomonas* aeruginosa is associated with poorer health in cystic fibrosis patients: an Irish registry analysis. BMC Pulm Med 2017;17(1):70.
[7] Coughlan CA, Chotirmall SH, Renwick J, Hassan T, Low TB, Bergsson G, et al. The effect of *Aspergillus* fumigatus infection on vitamin D receptor expression in cystic fibrosis. Am J Respir Crit Care Med 2012;186(10):999–1007.
[8] Mortensen KL, Johansen HK, Fuursted K, Knudsen JD, Gahrn-Hansen B, Jensen RH, et al. A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and antifungal susceptibility. Eur J Clin Microbiol Infect Dis 2011;30(11):1355–63.
[9] Ronchetti K, Tame JD, Paisey C, Thia LP, Doull I, Howe R, et al. The CF-Sputum Induction Trial (CF-SpIT) to assess lower airway bacterial sampling in young children with cystic fibrosis: a prospective internally controlled interventional trial. Lancet Respir Med 2018;6(6):461–71.
[10] Maiya S, Desai M, Baruah A, Weller P, Clarke JR, Gray J. Cough plate versus cough swab in patients with cystic fibrosis; a pilot study. Arch Dis Child 2004;89(6):577–9.
[11] Guegan H, Chevrier S, Belleguic C, Deneuville E, Robert-Gangneux F, Gangneux JP. Performance of molecular approaches for *Aspergillus* detection and azole resistance surveillance in cystic fibrosis. Front Microbiol 2018;9:531.
[12] Valenza G, Tappe D, Turnwald D, Frosch M, Konig C, Hebestreit H, et al. Prevalence and antimicrobial susceptibility of microorganisms isolated from sputa of patients with cystic fibrosis. J Cyst Fibros 2008;7(2):123–7.
[13] Baxter CG, Dunn G, Jones AM, Webb K, Gore R, Richardson MD, et al. Novel immunologic classification of aspergillosis in adult cystic fibrosis. J Allergy Clin Immunol 2013;132(3):560–6 e10.
[14] Reece E, McClean S, Greally P, Renwick J. The prevalence of *Aspergillus* fumigatus in early cystic fibrosis disease is underestimated by culture-based diagnostic methods. J Microbiol Methods 2019;164:105683.
[15] Baxter CG, Jones AM, Webb K, Denning DW. Homogenisation of cystic fibrosis sputum by sonication–an essential step for *Aspergillus* PCR. J Microbiol Methods 2011;85(1):75–81.
[16] Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. *Pseudomonas* aeruginosa and other predictors of mortality and morbidity in young children with cystic fibrosis. Pediatr Pulmonol 2002;34(2):91–100.
[17] Briard B, Mislin GLA, Latge JP, Beauvais A. Interactions between *Aspergillus* fumigatus and pulmonary bacteria: current state of the field, new data, and future perspective. J Fungi 2019;5(2) (Basel).
[18] Mac Aogain M, Narayana JK, Tiew PY, Ali N, Yong VFL, Jaggi TK, et al. Integrative microbiomics in bronchiectasis exacerbations. Nat Med 2021;27(4):688–99.
[19] Cuthbertson L, Felton I, James P, Cox MJ, Bilton D, Schelenz S, et al. The fungal airway microbiome in cystic fibrosis and non-cystic fibrosis bronchiectasis. J Cyst Fibros 2021;20(2):295–302.
[20] C.F. Trust. Laboratory standards for processing microbiological samples from people with cystic fibrosis on behalf of The UK Cystic Fibrosis Trust Microbiology Laboratory Standards Working Group. 2010 10th March 2021; 1st Edition. Available from: https://www.cysticfibrosis.org.uk/sites/default/files/2020-12/Laboratory%20standards.pdf.
[21] Lightly TJ, Phung RR, Sorensen JL, Cardona ST. Synthetic cystic fibrosis sputum medium diminishes Burkholderia cenocepacia antifungal activity against *Aspergillus* fumigatus independently of phenylacetic acid production. Can J Microbiol 2017;63(5):427–38.
[22] McDaniel MS, Schoeb T, Swords WE. Cooperativity between stenotrophomonas maltophilia and *Pseudomonas* aeruginosa during polymicrobial airway infections. Infect Immun 2020;88(4) e00855–e00819.
[23] Bottery MJ, Matthews JL, Wood AJ, Johansen HK, Pitchford JW, Friman VP. Inter-species interactions alter antibiotic efficacy in bacterial communities. ISME J 2022;16(3):812–21.
[24] Hotterbeekx A, Kumar-Singh S, Goossens H, Malhotra-Kumar S. *In vivo* and *In vitro* Interactions between *Pseudomonas* aeruginosa and Staphylococcus spp. Front Cell Infect Microbiol. 2017;7:106.
[25] Ferreira JA, Penner JC, Moss RB, Haagensen JA, Clemons KV, Spormann AM, et al. Inhibition of *Aspergillus* fumigatus and Its Biofilm by *Pseudomonas* aeruginosa is dependent on the source, phenotype and growth conditions of the bacterium. PLoS ONE 2015;10(8):e0134692.
[26] Sass G, Nazik H, Penner J, Shah H, Ansari SR, Clemons KV, et al. Studies of *Pseudomonas* aeruginosa mutants indicate pyoverdine as the central factor in inhibition of *Aspergillus* fumigatus biofilm. J Bacteriol 2018;200(1) e00345–e00317.
[27] Trunk K, Peltier J, Liu YC, Dill BD, Walker L, Gow NAR, et al. The type VI secretion system deploys antifungal effectors against microbial competitors. Nat Microbiol 2018;3(8):920–31.
[28] Delhaes L, Touati K, Faure-Cognet O, Cornet M, Botterel F, Dannaoui E, et al. Prevalence, geographic risk factor, and development of a standardized protocol for fungal isolation in cystic fibrosis: results from the international prospective study "MFIP". J Cyst Fibros 2019;18(2):212–20.
[29] Henry T, Iwen PC, Hinrichs SH. Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. J Clin Microbiol 2000;38(4):1510–15.
[30] de Heer K, Kok MG, Fens N, Weersink EJ, Zwinderman AH, van der Schee MP, et al. Detection of airway colonization by *Aspergillus* fumigatus by use of electronic nose technology in patients with cystic fibrosis. J Clin Microbiol 2016;54(3):569–75.
[31] Bessonova L, Volkova N, Higgins M, Bengtsson L, Tian S, Simard C, et al. Data from the US and UK cystic fibrosis registries support disease modification by CFTR modulation with ivacaftor. Thorax 2018;73(8):731–40.
[32] Lipuma JJ. The changing microbial epidemiology in cystic fibrosis. Clin Microbiol Rev 2010;23(2):299–323.
[33] Yousfi H, Ranque S, Rolain JM, Bittar F. *In vitro* polymyxin activity against clinical multidrug-resistant fungi. Antimicrob Resist Infect Control 2019;8:66.
[34] Schwarz P, Djenontin E, Dannaoui E. Colistin and isavuconazole interact synergistically *in vitro* against *Aspergillus* nidulans and *Aspergillus* niger. Microorganisms 2020;8(9):1447.
[35] Harun SN, Holford NHG, Grimwood K, Wainwright CE, Hennig S. Australasian cystic fibrosis bronchoalveolar lavage study g. *Pseudomonas* aeruginosa eradication therapy and risk of acquiring *Aspergillus* in young children with cystic fibrosis. Thorax 2019;74(8):740–8.
[36] Duesberg U, Wosniok J, Naehrlich L, Eschenhagen P, Schwarz C. Risk factors for respiratory *Aspergillus* fumigatus in German cystic fibrosis patients and impact on lung function. Sci Rep 2020;10(1):18999.
[37] Burns JL, Van Dalfsen JM, Shawar RM, Otto KL, Garber RL, Quan JM, et al. Effect of chronic intermittent administration of inhaled tobramycin on respiratory microbial flora in patients with cystic fibrosis. J Infect Dis 1999;179(5):1190–6.
[38] Bargon J, Dauletbaev N, Kohler B, Wolf M, Posselt HG, Wagner TO. Prophylactic antibiotic therapy is associated with an increased prevalence of *Aspergillus* colonization in adult cystic fibrosis patients. Respir Med 1999;93(11):835–8.
[39] Hong G, Psoter KJ, Jennings MT, Merlo CA, Boyle MP, Hadjiliadis D, et al. Risk factors for persistent *Aspergillus* respiratory isolation in cystic fibrosis. J Cyst Fibros 2018;17(5):624–30.