Resistome analyses of sputum from COPD and healthy subjects reveals bacterial load-related prevalence of antimicrobial-resistance-encoding genes.

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Abstract

Background: Antibiotic resistance is a major global threat. We hypothesised that the chronic obstructive pulmonary disease (COPD) airway is a reservoir of antimicrobial resistance genes (ARGs) that associate with microbiome-specific COPD phenotypes.

Methods: Resistomes were determined by quantitative PCR targeting 279 specific ARGs in sputum samples obtained from subjects with COPD at stable, exacerbation and recovery visits (n=55; COPD-BEAT study) or healthy controls with (n=7) or without (n=22) exposure to antibiotics in the last 12 months (EXCEED study) and bronchial brush samples from subjects with COPD (n=8) and healthy controls (n=7) (EvA study).

Findings: The ARG mean [SEM] prevalence was increased in sputum in stable COPD 55 [2]) versus healthy controls (29 [2]; p=0.002). ARG prevalence correlated with total bacterial abundance (r^2=0.23; p<0.001). The antibiotic-resistant gene prevalence was not related to COPD symptoms and lung function nor their change at exacerbation. In the COPD subgroup with high Gammaproteobacteria-associated exacerbations compared to high Firmicutes subgroup the ARG prevalence was not different at stable state but significantly declined from stable through exacerbation to recovery (p=0.011) without changes in total bacterial abundance. The ARG patterns were similar in COPD versus health, COPD microbiome-subgroups and between sputum and bronchscopic samples independent of antibiotic exposure in the last 12 months.

Interpretation: ARGs are highly prevalent in sputum, broadly in proportion to bacterial abundance in both healthy and COPD subjects. Thus COPD appears to be an ARG reservoir due to high levels of bacterial colonisation.

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Introduction

The ever increasing prevalence of antimicrobial resistance genes (ARGs) in bacteria detected in clinical samples is a global threat[1] encompassed conceptually in the term "resistome"[2]. While the gut microbiota have been extensively studied in this regard[3,4], there is little known about the respiratory bacterial community as a reservoir of ARGs in health and disease.

Antimicrobial resistance is a particular concern in chronic obstructive pulmonary disease (COPD) and other chronic respiratory conditions where these agents are recommended for both acute and prophylactic treatment[5]. While resistance may present a challenge to management, there is clear potential for such patients to become a reservoir for the dissemination of ARGs to the wider community[6]. It will be important to characterise the nature and extent of ARGs in chronic lung disease to develop appropriate antimicrobial stewardship responses to this threat.

We and others have sought to better understand the course of COPD and the nature of exacerbations through microbiome analyses applied to sputum samples[7-10]. In a well-studied local COPD cohort [11] (designated BEAT), we recently showed that the ratio between Gammaproteobacteria and Fimicutes (G:F ratio) in sputum samples identified an apparently antibiotic responsive subgroup of exacerbations associated with an increase in G:F at exacerbation which returned to baseline on recovery[12]. This

subgroup was designated HG, while the numerically dominant HF subgroup showed no significant disturbance in G:F through exacerbation to recovery. Intriguingly, the HF subgroup had suffered twice the frequency of exacerbations in the preceding year than their HG counterparts. We therefore hypothesised that differences in ARG profiles between the HF and HG subgroups might account for these patterns.

To investigate our primary hypothesis that COPD patients constitute an important reservoir for ARGs, we undertook high-throughput quantitative PCR targeting specific ARGs in bacterial DNA extracts from sputum samples obtained from the BEAT cohort and compared these to extracts from induced sputum samples from broadly age matched health volunteers (EXCEED study). Use of serial BEAT samples extending through exacerbation events allowed us also to investigate dynamic changes in ARG levels occurring at exacerbation and any specific relationships to the HG and HF subgroups. ARG profiling of bronchial brush samples from additional COPD patients (EvA study) allowed us to explore ARG prevalence in samples free from contamination by the oropharyngeal microbiota. To our knowledge we report here the first airway 'resistomics' study to explore and classify the type and abundance of ARGs present in the respiratory tract in COPD and in health.

Material and Methods

Subjects and samples

Airway samples were obtained from 3 independent studies [11,13,14]. In the BEAT and EXCEED studies subjects were recruited to a single centre in Leicester, UK, while EvA subjects were recruited as part of a multi-centre European study. BEAT samples studied here were taken at consecutive stable, exacerbation onset (prior to antibiotics) and 6 weeks post exacerbation onset, visits. The study design and endpoints have been described previously [11,14]. Sputum samples were available from 55 subjects at each visit. The EXCEED study is a regional genetic epidemiology study consented for recall based upon genotype and phenotype. Sputum samples from 29 healthy controls were obtained from this study; seven subjects gave a history of antimicrobial exposure (any reason) in the preceding 12 months. The EvA study design and endpoints have been described previously [13]. DNA extracts from bronchial brush samples were available from 22 subjects with COPD and 7 healthy controls. All subjects provided written informed consent and local ethics approvals were obtained for each study (08/H0402/19).

DNA extraction from sputum and bronchial brush samples

For the BEAT and EXCEED studies QIAamp DNA Mini Kit ("Gram positive bacteria extraction"; Qiagen, California, USA) was used to extract the DNA from the homogenized sputum. For the bronchial brush samples (EvA) the Qiagen "AllPrep DNA/RNA Mini Kit" was used to extract DNA and total RNA following the manufacturer's protocol. Molecular grade water (Sigma) was used for negative controls in each extraction batch. The DNA was stored at -20°C until subsequent analysis.

High-throughput quantitative PCR (HT-qPCR)

The Wafergen SmartChip Real-time PCR system (WaferGen Inc., USA) was used to perform high-throughput qPCR reactions at the Plateforme Génomique Environnementale et Humaine (GEH) in Université de Rennes 1, France. A custom set of 296 validated primer pairs targeting 283 ARGs, 8 transposase genes and 2 integron-integrase genes and the 16S rRNA were used [15]. In this study, two new validated primer pairs were included targeting β-globin and Actin in order to obtain information about the background human DNA in each sample [16,17]. Four ARG primer pairs were continually positive in the non-template control and were therefore discarded from the analysis.

The PCR mixture for this assay consisted of 50 nl of 2×LightCycler 480 SYBR® Green I Master Mix (Roche Inc., USA), 10 nl of each forward and reverse primers with the final concentration of 1 μM, 20 nl of DNAse and RNAse free distilled water and finally 10 nl of DNA template making the final reaction volume 100 nl. A non-template control was included per chip. Cycling conditions were described previously [18]. Samples with multiple melting peaks as well as amplification efficiency beyond the range 1.8-2.2 were discarded. A threshold cycle (Ct) less than 31 was used as the detection limit. The rationale for the selected detection limits is explained in the online supplement along with data showing that PCR signals for genes generally encoded in the same genomic regions are correlated.

Absolute quantification of 16S rDNA

The absolute 16S rRNA gene copy number was calculated separately using Roche 480 (RocheInc., USA) by standard curve method as described previously [19]. *Moraxella catarrhalis* genomic DNA prepared in 10-fold dilution series ranging from 10^7 to 10^2 copies/µl, were used as standards. The relative copy number of ARGs generated by the HT- qPCR (was calculated refereeing to a previous study[19]) could be transformed to absolute copy number by normalizing to absolute 16S rRNA gene copy number.

Statistical analysis

Data processing was done with Microsoft Excel 2013 (Microsoft Office 2013, Microsoft, USA). Graphs and statistical analysis were performed using GraphPad Prism (Version 7, San Diego, CA). Parametric and nonparametric data are presented as mean (SEM) and median (inter- quartile range) respectively. Correlations were determined by linear regression. Unpaired student t-tests and one way analysis of variance (ANOVA) were used to compare continuous parametric data between groups. Likewise, the paired t- test and repeated measures analysis of variance (RM-ANOVA) were performed for within-group comparisons. For statistical significance $p<0.05$ was applied. Heatmap graphs were produced using R studio with Pheatmap package.

Results

The clinical characteristics of the subjects from the COPD-BEAT, EXCEED and EvA cohorts are shown in **Table 1**. While not matched, key characteristics overlap and allow a basis for comparison.

We provide raw Ct results for all our targets and samples in the online supplement.

Sputum ARG prevalence in COPD (BEAT) and healthy volunteers (EXCEED)

Overall analysis of sputum DNA extracts representing the exacerbation episodes of 55 subjects from the BEAT study and 29 samples from the EXCEED (healthy volunteer study) revealed positive signals respectively for 211 and 115 of the 279 targets analysed. The results for stable BEAT (n=55) and EXCEED samples from subjects without (n=22) and with (n=7) prior antibiotic exposure are illustrated in figures 1A and 1B. The proportions of positive signals associated with resistance to different antibiotic classes were similar between the three groups with minor differences in the antibiotic exposed EXCEED subjects, likely reflecting the low number in this group (Figure 1A). ARGs detected per subject are compared between the groups in Figure 1B and show overlapping distributions. While the median ARG positive frequency was significantly higher in the BEAT group compared to EXCEED (37 vs 27, $p=0.0019$) the mean bacterial burdens (16S) were also significantly lower in the latter group ($3.5x10^8$ vs $5.8x10^7$, $p<0.0001$).

Heatmaps showing the prevalence of positive signals for ARGs related to agents regularly used in the management of COPD exacerbations (β-lactams, macrolides, and tetracyclines) and their abundances for the subjects' stable stage samples are shown in Figures 1C and 1D. While strong positive signals are apparent in all four groups, clustering does not separate EXCEED from BEAT samples on the basis of the specific targets quantified, particularly after normalisation to bacterial burden (Figure 1D). Overall ARG prevalence per sample was significantly ($p<0.0001$) correlated ($r^2= 0.019$) with bacterial burden (Figure 2) with a greater increment per increase in bacterial burden associated with healthy subjects.

Exacerbation-associated changes in the BEAT group

To determine whether COPD exacerbations were associated with ARG prevalence, we tested for correlations with the clinical metadata collected previously [11]. No significant correlations were found apart from with FEV_1 and mCRQ between exacerbation and recovery samples in patients receiving β-lactams just attained significance ($p<0.5$; table S2).

We next compared ARGs detected in the COPD subgroups characterised by high Gammaproteobacteria (HG; n=20) and high Firmicute (HF; n=35) sputum microbiomes (Figure 3). ARG prevalence was similar in both subgroups with median detections ranging from 32 to 39 positives per sample (range 4-74). A trend towards decline in the number of ARGs detected per subject through the samples taken at stable, exacerbation and

recovery stages was apparent in HG exacerbations ($p=0.01$, $r^2=0.22$) but not with HF, while the median bacterial burden measured by 16S rDNA assay was stable and equivalent in both groups. As previously reported[12], antimicrobial treatment between the exacerbation and recovery samples was associated with a significant ($p<0.0001$) decline in the G:F ration in the HG, but not in the HF group (Figure 3A).

The distribution of detected ARGs across different antimicrobial targets was similar in the stable samples from both subgroups (Figure 3B). This lack of difference is further substantiated in the heatmap and cluster analysis of 16S normalised abundance measures for β-lactam-, macrolide-, multidrug- and tetracycline- associated ARGs (Figure 3C). However, review of the correlations between ARG prevalence and 16S signals revealed that, while the HF group showed significant correlation, the HG group did not, both across all samples and individually in the stable, exacerbation and recovery samples (Figure 3D).

Treatment with antibiotics generally enriches the relative abundance of bacteria expressing ARGs directed against the cognate agent. Review of our quantitative ARG data in relation to the treatment given (co-amoxiclav, amoxicillin or doxycycline) did not reveal any clear evidence that this occurred in BEAT patients (Figure S2). While significant variation across the three sample times was seen for several comparisons between the agent given and signals for specific ARGs, there was no consistent evidence of selection for particular determinants.

To determine whether the occurrence and abundance of any ARG was associated with particular signals in the microbiome analyses we have previously performed on these samples[15], we looked for correlations between ARG signals and the proportion of particular genera in each sample; only the 30 most abundant genera were included (>1% of reads across all samples). Taking a correlation coefficient (r^2) of >0.15 and $p<0.01$ the associations detected are shown in Table 2. Of note are four associations with *Pseudomonas*, including a carbapenemase (*cphA-01*) and the surprising association with *vanB* which is described in *Enterococcus.* Three negative associations were also detected, indicating that the cognate ARG was reduced or absent in relation to presence or relative abundance of the genus identified. The association of *pbp2x* (associated with increased penicillin MICs) positive samples with *Streptococcus* is also noteworthy. Applying the Bonferoni false discovery correction ($q<0.01$), only the xxxx associations remain significant.

Bronchial brush (EvA) sample analyses

Sputum samples are subject to contamination with the microbiota in the oropharynx. As part of the EvA study, we were provided with eukaryote targeted DNA extracts from bronchial brush samples taken from 8 stable COPD patients and 7 healthy volunteers and these yielded 57 and 37 positive ARG signals respectively). These results reflect lower extraction efficiency (~10-fold) and some species bias with the eukaryotic DNA extraction protocol and we characterise this in the online supplement

(Figure S4). Notwithstanding this lower efficiency, the pattern of results obtained was broadly similar to those described above for the BEAT and EXCEED groups (Figure 4). We note that, despite the low sample numbers, the ARG:16S relationship was significant in the control group and, as before, demonstrated a steeper gradient than the COPD group (Figure 4C).

Comparison of specific ARG prevalence across the three studies and subgroups.

Table 3 shows a comparison between the per cent prevalence of positive ARG signals relevant to different antimicrobials in all three cohorts. The table is ordered according to the prevalence found in the EXCEED group. The 10 most frequently detected ARGs are almost identical in the three cohorts. Two targets, *intI1* and *pbp2x*, were detected at least 20% more frequently in BEAT over EXCEED samples. Conversely, four targets, *IS613, tet(32), pncA* and *ceoA* were found in similar excess in the EXCEED samples.

Comparison of the ARG prevalence between the HG and HF subgroups, including all the detected ARGs across the two groups (211), revealed a mean excess of 1.12% of positive signals per sample in the HF group ($p=0.0017$). A few examples are highlighted in table 3, however, this result should be viewed with caution since more HF than HG samples were assessed (105 vs. 60).

The detection frequencies (38-0%) of targets generally considered to be of particular clinical concern (extended spectrum β-lactamases (ESBLs),

AmpC β-lactamases, carbapenemases, methicillin resistance (*mecA*) and proteins associated with vancomycin resistance) are shown in Table 4. For comparison, results for the narrow spectrum β-lactamases detected by the broad specificity blaTEM primers are included; these detect much of the TEM family from which many ESBL enzymes are derived. This target was prevalent in healthy subjects, modestly more so in COPD sputum and bronchial brush samples but was not detected in control samples from the EvA study. Overall there was a modest excess of positive signals for ESBL-encoding ARGs in the COPD samples. Primers directed to the AmpC class of generally inducible β-lactamases again showed a modest excess in BEAT over EXCEED samples although the *ampC-04* directed primers showed an excess in the latter group. *MecA* and *vanB* were exclusively detected in BEAT samples. Several carbapenemase-encoding ARGs, including *NDM1* (one low positive), were also detected.

Discussion

Patients with COPD suffering exacerbations are almost universally given antibiotics. We therefore hypothesised that their sputum microbiota would constitute an important reservoir for antimicrobial resistance-encoding genes. With a view to enabling evidence-based antimicrobial stewardship, we have undertaken the first substantial survey of resistomes present in sputum from COPD and healthy control subjects.

ARGs were found to be more prevalent in sputum samples from COPD than

those from healthy volunteers with a mean prevalence 37% higher in the former. However, this excess was predominantly attributable to the bacterial burden in sputum. Thus, with the exception of a limited set of differentially detected ARGs, COPD patients only appear to constitute important ARG reservoirs inasmuch as their sputum bacterial burden is higher than that found in healthy individuals; the most prevalent ARGs were common across the sample sets. Linear regression analysis showed that for every tenfold increase in 16S signal, detection of 6 additional ARGs could be expected in COPD patients, while the equivalent figure would be 14 for healthy volunteers (Figure 1C). Perhaps, as bacterial communities in sputum approach saturation, capacity to accommodate more ARGs is somehow limited.

We next interrogated data from the 55 separate COPD exacerbation events in our samples from the BEAT study for relationships between our ARG results and our exacerbation-related metadata. Apart from modest evidence that improvements in FEV_1 and mCRQ between exacerbation and recovery samples were associated with reductions in ARG prevalence, we found no clear relationships with patient clinical status.

Comparison of our HG and HF subgroups revealed that, while the distribution of ARG determinants was similar, prevalence declined in the former but not the latter. We note the increase in G:F ratio at exacerbation and return to stable levels in the HG subgroup compared to its stability in the HF subgroup and suggest, as previously, that the differences observed may reflect efficacy of antimicrobial treatment in patients with an HG

profile. As a potential explanation for the stability of the G:F ratio in the HF subgroup, we hypothesised that this subgroup might harbour higher rates of ARGs. The 12% excess of positive ARG signals in the HF group provides modest evidence consistent with this proposal.

We found no evidence that antibiotic administration enriched ARG prevalence through exacerbations. A possible explanation for this is that such enrichment may be short lived and that it was missed in limiting our analyses to a six week post exacerbation sputum sample. There is good evidence that repeated use of antibiotics in COPD does increase the prevalence of phenotypic resistance. The lack of evidence for this in our BEAT study population may reflect the low sensitivity of routine sputum culture. Such samples are extensively diluted in nationally accredited diagnostic culture procedures, while many COPD studies have used analysis of undiluted samples to look for resistant colonies.

We do not know whether the ARGs detected were expressed in our samples nor do we know their bacterial hosts. However, correlation between the ARG signals and genera detected in microbiome analyses of the same samples, revealed expected correlations with *Pseudomonas* and *Streptococcus* but also several unexpected associations. Bonferoni correction revealed that false discovery may have been responsible for some of these and the associations would require confirmation before further investigation is warranted.

The prevalence of *pbp2x* signals, which were more frequent in the BEAT

samples, indicates potential for pneumococcal penicillin resistance. However, the related altered penicillin binding protein has been extensively detected in *Streptococcus mitis*, which is generally regarded as non-pathogenic in the lung. Nonetheless, there is clear potential for the naturally transformable *S. pneumoniae* to acquire pbp2x from this source.

We investigated a small number (15) of bronchial brush samples from the EvA study here as an initial assessment of the degree to which upper airway contamination may have contributed to our sputum results. Although the bacterial DNA yield was sub-optimal and biased against Gram-positive organisms, the most frequently detected ARGs were essentially the same as those found in sputum and there were no robust differences between the COPD and control samples. A larger sample set comparing brush and sputum samples subjected to identical analytical procedures would be required to securely differentiate upper and lower airway sources of the ARG positive signals in sputum.

Taking into account the generally stable hierarchy of ARG frequencies observed across our three sample sets (table 3), we suggest that the probability of positivity for any given ARG in a sample will be related the bacterial burden. Thus an otherwise healthy individual with a 16S signal of 10^6 copies per gram in their sputum would have a roughly 40% probability that *blaTEM* would be detected in the same sample. Given that each sample represents a small fraction of the total pulmonary bacterial burden, extrapolation of these considerations to the whole lung microbiome

suggests that a low frequency ARGs with high clinical impact may be present in many healthy lungs.

Finally, we reviewed ARG signals relating to target genes considered sufficiently important to warrant detection of their presence or related phenotypes in most clinical microbiology laboratories. Here the evidence for differential occurrence in COPD was stronger, with modest to low frequencies of AmpC-, ESBL-, carbapenemase-, *mecA*- and *vanB*- encoding genes detected. Two carbapenemase, 3 ESBLs and 5 AmpC genes were also detected in the EXCEED control group. Patients from whom recognised pathogens expressing these ARGs are detected in screening or clinical specimens, would normally be managed by isolation. We have recently reported evidence that ARGs detected here (*ermB* and *mefA*) can also be detected in face-mask collected exhalations from COPD patients[34]. While such airborne dissemination may explain the frequency of ARG detection in our healthy volunteers, further studies will be needed to identify the risk that ARGs with high potential clinical impact may spread this way.

Does the presence or pattern of multiple ARGs in the sputum microbiome have any clinical consequences? Beyond the associations previously established for the HG subgroup (inflammatory markers and post-bronchodilator per cent predicted FEV_1[12]), we were not able to find any links between the ARG signals detected here and our clinical metadata for the BEAT subjects. It is likely that both expression of antimicrobial altering or degrading enzymes by "non-pathogens" and abundance of these organisms will reduce the bioavailability and impact of antimicrobials on key

pathogens. Further study will be needed to assess this possibility.

In this first survey of ARGs in the respiratory tract microbiome, several limitations must be taken into account. Positive ARG signals were assigned on the basis of a precautionary PCR cycle threshold (Ct) value in line with previous studies in this field (see supplementary methods) and, in the absence of ARG standards, quantitation was based on differences in Ct. Given the scale of the work (223 samples), we have focussed our analysis on multiple positive detections in place of confirmatory assays to assess rare positives. It could also be argued that we may have missed acute changes in the ARG profile as our exacerbation and recovery samples were taken before (day 0) and after (day 42) antimicrobial therapy. However, our main aim was to observe sustained changes.

While many of the ARGs targeted here are known to be encoded on mobile elements, many also reflect intrinsic resistance native to the host bacterial genome. Assessment of the threat of ARG dissemination from the respiratory microbiome would require a different approach from that taken here and could potentially be enabled by metagenomic analyses. Finally, the primer set deployed here has been established for several years. Numerous polymorphisms have been detected in the gene families concerned as well as recognition of novel ARGs and our coverage cannot be considered comprehensive. For example, our primer set would not have detected the carbapenemase encoded by *oxa48* that is regarded as high value in current clinical practice.

While the potential challenges posed by the bacterial resistome in the lung [20,21] and other body sites[22] have been discussed, we are not aware of work directly comparable to the present study. Some previous respiratory studies addressing the resistome have concentrated on samples from cystic fibrosis patients[23,24] and have attempted to reconcile detected resistance genes and phenotypic resistance in recognised pathogens as well as the intriguing potential of bacteriophages as vehicles of ARG dissemination[24,25]. Most resistome studies have focussed on faecal samples to assess the gut reservoir of ARGs and relationships to interchange with the local environment and geographic variations thereof[26-33]. While the general airborne microbiome may be a source of the ARGs we detected here, we have recently observed ARGs in exhaled breath from human subjects [34] and these potential sources require further investigation. Transit between body sites must also be considered and we note with interest recent application of the concepts of island biogeography in this context[35]. As recently reviewed[36], the key challenge remains translating human resistome analyses into clinical benefit.

In conclusion, we have found that while the human respiratory tract is a reservoir for numerous ARGs, their prevalence in samples appears more dependent on bacterial load than clinical condition; healthy subjects with similar ages to our COPD patients carried most of the same resistance determinants. Key further questions include understanding the relative contributions of antibiotic and environmental exposure to prevalence of ARGs and their influence on clinical outcomes.

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