Carbapenem-resistant Acinetobacter Baumannii Ventilator-Associated Pneumonia in Critically Ill Patients: Potential Inference with Respiratory Tract Microbiota Dysbiosis

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Research

Keywords: Carbapenem-resistant Acinetobacter baumannii, Ventilator-associated pneumonia, Multi-genomics analysis, Microbiome, Virulence gene.

DOI: https://doi.org/10.21203/rs.3.rs-736916/v1

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Abstract

Background: Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is a common cause of ventilator-associated pneumonia (VAP) in intensive care unit (ICU) patients, but infection and colonization are difficult to distinguish which aggravates the abuse of antimicrobial drugs and further accelerates the evolution of drug resistance. We sought to provide new clues for the diagnosis, pathogenesis and treatment of CRAB VAP based on lower respiratory tract (LRT) microbiota.

Methods: A prospective study was conducted on patients with mechanical ventilation from July 2018 to December 2019 in a tertiary hospital. Multi-genomics studies (16S rRNA amplicon, metagenomics and whole-genome sequencing [WGS]) of endotracheal deep aspirate (ETA) were performed.

Results: Fifty-two ICU patients were enrolled, including 24 with CRAB VAP (CRAB-I), 22 with CRAB colonization (CRAB-C), and six CRAB-negative patients (infection-free) (CRAB-N). Diversity of pulmonary microbiota was significantly lower in CRAB-I than in CRAB-C or CRAB-N (mean Shannon index, 1.79 vs. 2.73 vs. 4.81, P<0.05). Abundances of 11 key genera differed between the groups. *Acinetobacter* was most abundant in CRAB-I (76.19%), moderately abundant in CRAB-C (59.14%), and least abundant in CRAB-N (11.25%), but its interactions with other genera exhibited the opposite pattern. Metagenomics and WGS analysis showed that virulence genes were more abundant in CRAB-I than in CRAB-C. Multi-locus sequence typing (MLST) of 46 CRAB isolates revealed that the main types were ST208 (30.43%) and ST938 (15.22%), with no difference between CRAB-I and CRAB-C.

Conclusions: LRT microbiota dysbiosis including elevated relative abundance of *Acinetobacter* and reduced bacterial interactions, and virulence enrichment may lead to CRAB VAP.

Introduction

*Acinetobacter baumannii* (AB) is an environmental microorganism that can contaminate the surface of hospital equipment and colonise skin, wounds, and other parts of patients [1]. VAP is a frequent complication in ICUs and is associated with prolonged mechanical ventilation, longer ICU stay, and poorer outcomes [2]. AB is frequently isolated from the respiratory tract in patients with tracheal intubation, which is considered to be a high-risk factor for VAP [3–4]. Yin et al. found that the prevalence of AB was high (31.7%) among VAP pathogens in 15 teaching hospitals in China from 2007 to 2016 [5]. Due to the heavy use of broad-spectrum antibacterial agents in ICU patients, most AB strains were multi-drug resistant or even pan-drug resistant. A survey of hospital-acquired pneumonia (HAP) and VAP conducted from 2007 to 2013 revealed that multidrug-resistant AB (MDRAB) increased yearly and ranked first in some ICU bacterial lists [6].

The carbapenem-resistant AB (CRAB) genome encodes various drug-resistance genes and virulence factors (VFs), including efflux pumps, iron acquisition systems, secretion systems, phospholipases, and capsular polysaccharides, which help the bacterium survive antibiotic treatment and colonise in the environment [7–8]. Without effective therapy, the mortality of patients with nosocomial CRAB infection
remains high [5, 9]. Accordingly, the World Health Organization (WHO) has designated CRAB as a pathogen that poses a major threat to human health and that should be urgently targeted by new antibiotics [10].

A key step in containing CRAB is the rational use of antibiotics based on accurate diagnosis of infection. However, the challenge of differentiating CRAB colonisation and infection could lead clinicians to prescribe excessive broad-spectrum antibiotics, potentially promoting the occurrence of drug-resistant bacteria and their spread in hospitals. Therefore, clinical infection control requires reliable methods for distinguishing CRAB colonisation from CRAB infection. Reduction in LRT microbiota diversity or elevated abundance of certain strains may lead to infections [11–12]. For example, LRT microbiota diversity was lower in 13 infected patients than in healthy control patients, and pathogenic bacteria in four subjects was consistent with the dominant flora identified by 16S rRNA analysis [12]. Through 16S rRNA analysis of 263 samples, Emonet et al. found that the low relative abundance of species in oropharyngeal secretions during intubation was strongly associated with subsequent VAP [13].

However, many aspects of the relationship between respiratory microecology and infection remain unknown, and current research is focused mainly on chronic lung diseases [14–15]. Budden et al. reviewed recent advances in understanding the composition of the lung microbiome and found that bacteria, viruses, and fungi from the respiratory tract produce structural ligands and metabolites that interact with the host and alter the development and progression of chronic respiratory diseases [15]. Hakansson et al. suggested that a complex interplay between the host, environment, and properties of the colonising microorganisms determines disease development and severity [16]. Moreover, Roquilly et al. proposed that the diversity of the microbiome and mucosal immunity are associated with HAP [17]. Metagenomic analysis of microbiome structure and function will aid in understanding the pathogenesis and regulatory networks of AB during infection [14].

This is the first study to analyse and compare the characteristics of LRT microbiota of CRAB-negative, CRAB-colonised, and CRAB-infected patients using 16S rRNA, metagenomics, and whole-genome sequencing (WGS). We investigated whether VAP patients are associated with unique LRT microbiota to explore the pathogenesis of VAP at the level of the microbiota and provide an improved basis for clinical decision-making.

**Materials And Methods**

**Study design and patient enrolment**

This prospective study was conducted from July 2018 to December 2019 in the adult ICUs of the First Affiliated Hospital, College of Medicine of Zhejiang University, China. The patient inclusion criteria were as follows: (1) patient was mechanically ventilated and hospitalised in the ICU; (2) Acute Physiology and Chronic Health Evaluation (APACHE) II score was greater than 12; and (3) collection of endotracheal deep aspirate (ETA) specimens was possible. Exclusion criteria were as follows: (1) age < 18 years; (2) comorbidity of chronic lung disease or lung cancer; (3) hospital stay < 24 hours; and (4) co-infection with
other bacteria. VAP was defined by the criteria of the Centers for Disease Control and Prevention (CDC) of the United States based on clinical, laboratory, radiological, and microbiological data [18]. Patients meeting VAP criteria with positive culture for CRAB ETA were assigned to the CRAB VAP group (CRAB-I). Respiratory tract CRAB colonisation (CRAB-C) was defined as CRAB-positive culture from ETA without VAP. Control patients (CRAB-N) were patients with neither CRAB VAP nor CRAB colonisation. Approval was obtained from the ethical board of the hospital (reference number: 2016-458-1).

Patient sampling and bacterial isolation

The aspirate samples were transported to the microbiology laboratory within two hours. ETAs were inoculated with a calibrated loop (0.001 ml). A gram-stained smear was prepared for all specimens and examined microscopically. MacConkey agar [HiMedia Laboratory Pvt. Ltd. India] and incubated aerobically at 37°C overnight. *A. baumannii* was identified by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) analysis using a VITEK MS instrument (bioMérieux, US). The confirmed *A. baumannii* from clinical and sputum samples were stored at −80°C for further study. The resistance of *A. baumannii* to antibiotics, including Piperacillin/ tazobactam (TZP), Cefoperazone/sulbactam (CSL), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Ciprofloxacin (CIP), Levofloxacin (LVX), Imipenem (IPM), Meropenem (MEM), Trimethoprim/sulfamethoxazole (TMX/SXT), Amikacin (AMK), and Gentamicin (GEN), was analysed according to the recommendation of Clinical and Laboratory Standards (CLSI, 2018) [19]. Resistance to Tigecycline (TGC) and Polymyxin (POL) was examined by the broth microdilution method and evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [20]. *Escherichia coli* ATCC25922 was used as a quality control strain. CRAB was defined as AB strains that were non-susceptible to imipenem or meropenem.

Clinical data

Clinical data including demographic variables, length of ICU stay, length of hospital stay, comorbidities, previous invasive procedures (central line insertion, intubation, continuous renal replacement therapy, and surgery under general an aesthesia), as well as lengths and types of antibiotic treatments and severity of illness (Acute Physiology and Chronic Health Evaluation (APACHE II)), were recorded [21]. Data on the levels of serum markers for liver and renal function (e.g. bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), gamma glutamyl transpeptidase (γ-GT), urea, and creatinine) as well as those of blood biomarkers of infection other than core temperature (e.g. white blood cell (WBC) and CRP) were also collected.

16S rRNA amplicon and data analysis

Total genomic DNA was extracted from samples using the CTAB method. PCR amplification was performed, purified amplicons were pooled, and paired-end sequencing was carried out on an Illumina NovaSeq PE250 platform. After demultiplexing and trimming of the barcode and primer sequence using FLASH (V1.2.7) [22], the paired-end raw read data of each sample was acquired. Subsequently, quality control was carried out using Qiime [23–24] and effective tags were obtained for analysis. OTUs were
clustered with a 97% similarity cut-off using Usearch (Version 7.0) [25]. OTU sequences were taxonomically annotated using the Mothur (ref) method and SILVA database (ref) [26]. The abundance matrices at the levels of phylum, class, order, family, and genus were constructed for each sample. MUSCLE (Version 3.8.31) software was used for rapid multiple sequence alignment, and the phylogenetic relationships of all OTU representative sequences were obtained [27]. Finally, the data of each sample was normalised, and the sample with the least amount of data was used as the standard. Alpha diversity (Shannon and Simpson index) within a sample and Beta diversity (Bray–Curtis dissimilarity matrix) across samples were calculated using the phyloseq R package (version 1.32.0). PCoA and NMDS analysis based on Bray–Curtis dissimilarity was performed using the Vegan R package (version 2.5.6) and visualised using the ggplot2 R package (version 3.3.2). ANOSIM was used to determine statistical significance. A complete list of sample names and accession numbers is provided in Table S1.

Metagenomics sequencing and analysis

Following fragmentation of microbial DNA, metagenomic sequencing was performed on an Illumina NovaSeq 6000. After paired-end Illumina sequencing, we employed a previously reported bioinformatics pipeline to detect and profile the airway microbiome (ref). The low-quality sequences were filtered out or trimmed using PRINSEQ-lite (Version 0.19.3). And then de novo assembly was generated using SPAdes genome assembler (Version 3.11.1) [28] and coding sequences were predicted using MetaGeneMark (Version 3.38). Taxonomy assignments of both the clean reads and coding sequences were performed by Kaiju classifier (Version 1.7.2) with the National Center for Biotechnology Information Refseq database [29]. The functions of coding sequences were obtained using DIAMOND software (Version 0.9.30) with Kyoto Encyclopedia of Gene and Genomes (KEGG) database [30–31]. Furthermore, virulence genes in metagenomics sequences were identified by comparing the coding sequences against the Virulence Factor Database using DIAMOND software [32]. The correlation coefficient between bacteria and virulence genes was generated using Python-based SparCC tool with SparCC correlation method and visualized using Cytoscape (Version 3.8.0). We performed multivariate linear regressions with feature selection, using the lasso penalized maximum likelihood technique in the “glmnet” R package (Version 4.0.2). A complete list of sample names and accession numbers is provided in Table S1.

WGS and analysis

The genomic DNA of 46 isolates was extracted using a Qiagen DNA purification kit (Qiagen, Hilden, Germany) and sequenced on an Illumina HiSeq 4000-PE150. For each isolate, de novo assembly of reads was performed using SPAdes genome assembler. The assembled genome sequences were annotated using Prokka (version 1.14.6) [33]. GenBank files produced from Prokka were converted to GFF format, and then subjected to Roary (version 3.11.2) [34] to obtain core genome sequences. A maximum-likelihood phylogenetic tree was constructed using RaxML software (version 8.2.12) with 1000 bootstraps replicates [35]. Average nucleotide identity (ANI) was calculated by using a pyani (version 0.2.10). ANI values above 95% between genomes of these isolates denote the same species [36]. MLST analysis were determined according to the Institute Pasteur scheme (MLST-IP) and Oxford Database
(MLST-OD) [37]. Clonal complexes were assigned by eBURST and were defined as single locus [38]. CC was defined as a group of STs sharing at least five or more identical loci among the seven housekeeping genes tested by goeBURST (goeburst.phyloviz.net). CCs were named according to the number of the predicted founder ST. Antimicrobial resistance genes (ARG) and AB virulence genes were identified by comparing genome assemblies against the ResFinder antibiotic resistance gene database using the Abricate software (version 0.8) and against the Virulence Factor Database using the DIAMOND software (version 0.9.30), respectively [39]. We determined the capsular polysaccharide (KL) and lipooligosaccharide outer core (OCL) synthesis of the \textit{A. baumannii} using Kaptive software (version 0.5.1) [40]. A complete list of strain names and accession numbers is provided in Table S1.

**Statistical analysis**

The Linear Discriminant Analysis Effect Size (LEfSe) program was used to identify taxa that differed consistently between sample types [41]. Network X, built on correlation coefficients obtained using the Python-based SparCC tool with the SparCC correlation method and visualised using Cytoscape (version 3.8.0), was used to explore and visualise the associations between microbial communities [42]. Normally distributed continuous variables were expressed as means ± standard deviation (SD) and compared using Student's t-test, whereas non-normally distributed continuous variables were expressed as median and interquartile range (IQR) and compared using Mann–Whitney U-tests. Categorical variables were compared by the $\chi^2$ test or two-tailed Fisher's exact test, as appropriate.

**Accession numbers**

All sequencing data during the current study are available in the Sequence Read Archive (SRA). Metagenomics data and the 16S rRNA gene data are under BioProject PRJNA 681291, and the WGS data under BioProject PRJNA 679997.

**Results**

**Characteristics of the study population**

A total of 101 patients were screened. According to the inclusion criteria, 64 patients were enrolled, and 52 patients completed the entire study protocol (Fig. 1): 24 with CRAB-I, 22 with CRAB-C, and six with CRAB-N. The CRAB-I and CRAB-C patients did not differ in terms of age, sex, or severity indices (APACHE II scores), but C-reactive protein (CRP) and 30 day mortality were higher in CRAB-I (Table S2). All CRAB isolates were highly resistant to all antibiotics except amikacin, polymyxin, and tetracycline (Fig. S1).

**Comparison of microbiota of ETA**

**Overview**
16S rRNA sequencing from a total of 52 ETA specimens revealed that the average Operation taxonomic units (OTU) numbers for CRAB-N, CRAB-C, and CRAB-I were 1427, 2422, and 2248, respectively. Rarefaction curves of numbers of observed OTUs per sample and group indicated that almost all OTUs present in each group were detected (Fig. S2). Three samples failed to sequence. The LRT microbiome was examined in the remaining 49 ETA specimens by shotgun metagenomic sequencing (Table S1). The 16S rRNA sequencing data identified 597 genera, of which 249 (41.2%) were also identified in the metagenomic data (Fig. S3, Fig. S4). All of the alpha diversity indices in the metagenomic analysis were higher than those in the 16S amplicon analysis (Fig. S5).

**CRAB-positive patients had reduced microbiota diversity**

Relative to CRAB-N, the diversity of pulmonary microbiota in the CRAB-C group was significantly reduced, and a further reduction was observed in the CRAB-I group (Shannon index in CRAB-N, -C, and -I: 4.80 ± 1.47, 2.73 ± 1.24, and 1.79 ± 0.95; Simpson index: 0.90 ± 0.08, 0.60 ± 0.23, and 0.40 ± 0.20, respectively; P < 0.05 for all biodiversity parameters; Fig. 2A and 2B). Moreover, principal co-ordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) analysis of the Bray–Curtis dissimilarity metric revealed that the composition of the microbiota between the three groups were quite different (P = 0.001 and Stress = 0.113; respectively; Fig. 2C and 2D). Analysis of similarity (ANOSIM) comparative analysis revealed that the differences between the three groups were higher than those within each group, indicating that the microbial community structure of the LRT microbiota in the three groups was significantly distinct (Table S3).

**Bacterial taxonomic characters in the three groups**

To obtain a global view of LRT microbiota in the study subjects, we compared the taxa at the phylum and genus levels between the three groups by 16S rRNA amplicon analysis. Overall, Proteobacteria (43.57%), Firmicutes (2.58%), and Bacteroidetes (2.42%) were the dominant phyla in the 52 ETA specimens (Fig. 3A). The relative abundance of Proteobacteria was significantly higher in the CRAB-I and CRAB-C groups than in the CRAB-N group (91.77% vs. 84.50% vs. 49.25%, P < 0.05), and Firmicutes exhibited the opposite pattern (2.23% vs. 4.38% vs. 15.67%, P < 0.05) (Fig. 3B). At the genus level, the bacterial composition of the CRAB-positive groups differed from that of the negative group. *Acinetobacter* was the predominant genus in CRAB-I and CRAB-C patients (76.19% vs. 59.14%), followed by *Klebsiella* (5.80% vs. 8.01%) and *Pseudomonas* (2.84% vs. 6.88%). By contrast, in the CRAB-N group, the top three genera were *Acinetobacter* (11.24%), *Haemophilus* (8.67%), and *Pseudomonas* (9.68%) (Fig. 3C and 3D).

LEfSe comparison identified 11 significant biomarker genera (Fig. 3E) with discrimination value in the three groups. Further pairwise comparison revealed two significant biomarker genera (*Acinetobacter* and *Nocardia*) between the CRAB-C and CRAB-I groups (Fig. 3F), and the CRAB-I group had 14 significant biomarker genera with the CRAB-N group: *Acinetobacter* was enriched in the CRAB-I group and 13 other genera, including *Haemophilus, Bacteroides*, and *Streptococcus*, were enriched in the CRAB-N group...
(Fig. 3G). In addition, five key genera (Acinetobacter, unidentified Corynebacteriacea, Nesseria, Nordella, and Streptococcus) differentiated between the CRAB-C and CRAB-I groups (Fig. 3H).

Identifying potential microbial interactions by Correlation Network Analysis

Overall, the microbial co-occurrence network constructed from CRAB-I had a lower complexity index than those of CRAB-C and CRAB-N at the genus level (4239.37 vs. 4257.25 vs. 7459.00) (Fig. 4). The total number of negative microbial interactions, as indicated by the number of edges between the nodes, was highest in CRAB-N, moderate in CRAB-C, and lowest in CRAB-I (CRAB-N: n = 2356, 2189 positive and 167 negative; CRAB-C: n = 1436, 1411 positive and 25 negative; CRAB-I: n = 1533, 1516 positive and 17 negative). Notably, in the CRAB-I group, Acinetobacter was abundant and significantly negatively correlated with four genera (Klebsiella, Pseudomonas, unidentified Erysipelotrichaceae, and Oscillibacter), whereas in the CRAB-C group, it was negatively correlated with five other members of the microbiota (Limnobacter, Brevundimonas, Dialister, Barnesiella, and Bilophila), and in the CRAB-N group, it had six negative connections. Thus, the number of negative interactions decreased much more between CRAB-N and CRAB-I than the number of positive interactions.

Comparison of functional profiles of microbiota

LEfSe analysis using a logarithmic LDA score cut-off of 2.5 identified 46 and 55 different pathways in CRAB-C and CRAB-I, respectively, relative to CRAB-N, of which 40 and 45, respectively, increased. These pathways included fatty acid degradation, oxidative phosphorylation, nicotinate and nicotinamide metabolism, transport, porphyrin and chlorophyll metabolism, benzoate degradation, and biofilm formation in Vibrio cholerae (Fig. 5A and 5B). Moreover, signalling proteins (KEGG pathway ko99995, Fig. 5C) were more active in CRAB-C than in CRAB-I.

Comparison of VFs in the microbiome

In the three groups of patients, we detected a total of 1,628 virulence genes, divided into 69 functional groups. The top 10 most abundant functional virulence groups in each group accounted for 26.5% of all factors, and the composition of the “toxicity” functional group differed between the three groups of patients (Fig. 6A). These factors tended to be involved in iron uptake, siderophore biosynthesis, immune evasion, and biofilm formation. Ninety-five virulence genes (relative abundance > 0.1% for each) differed in abundance between CRAB-N and CRAB-C, and 105 differed between CRAB-N and CRAB-I (Table S4). Eleven genes, including AB57_0984, AB57_0990, AB57_0992, and mymA, were more abundant in CRAB-I than in CRAB-C (Fig. 6B and Table S4). Virulence gene networks constructed from CRAB-I had a higher complexity index than those from CRAB-C and CRAB-N patients (56206.99 vs. 44722.75 vs. 11052.12) (Fig. 6C). More virulence genes associated with Acinetobacter were detected in CRAB-I than in CRAB-C and CRAB-N, with functions including immune evasion, iron uptake, and VFDB-unclassified (Fig. 6D). Consistent with this association, the levels of VFs and the relative abundances of genera (Acinetobacter and Methylorubrum) or species exhibited a strong and significant positive correlation (R2 = 0.529, P =
1.1e-06; R2 = 0.755, P = 7.6e-12, Pearson’s correlation; Fig. 7A and 7B), indicating that differences in the abundance of VFs were driven by differences in the species present in each group of patients.

Whole-genome analysis of CRAB

The average nucleotide identity (ANI) of the 46 CRAB strains was > 95%, indicating that they belonged to the same species (Table S1, Fig. S6A). MLST of 46 CRAB was dominated by ST208 (30.43%), followed by ST938 (15.22%). eBURST analysis revealed that seven ST types (87.5%) clustered in the same clonal complexes (CCs) (CC92) (Fig. S6B). The KL types were mainly KL9, KL2, KL93, and KL7, and all strains belonged to the OCL1 type (Fig. 8). We observed no statistically significant difference in ST or KL type between the CRAB-I and CRAB-C groups (P = 0.478 and 0.444; respectively).

All CRAB isolates harboured \( \text{bla}^{\text{ADC}-25}, \text{bla}^{\text{OXA}-23}, \text{and} \ \text{bla}^{\text{OXA}-66} \). All isolates harboured more than one oxacillinase gene, and 28 (60.8%) harboured the class A \( \beta \)-lactamase gene \( \text{bla}^{\text{TEM}} \). The number of resistance genes did not differ significantly between the two groups (Fig. 8). Annotation and analysis of virulence genes showed that strains from CRAB-I had more virulence genes than those from CRAB-C (Fig. 8), and chi-square tests (Table 1) revealed that AB57_0990, LpxL, and ABZJ_00085 were more abundant in CRAB-I, consistent with the metagenomics analysis (Fig. 6B).

**Table 1.** The Virulence genes with significant Difference between infection and colonization groups of Carbapenem-resistant *Acinetobacter baumannii*.

| VFs       | Function  | CRAB-C (n = 22) | CRAB-I (n = 24) | Chi states | P values |
|-----------|-----------|-----------------|-----------------|------------|----------|
| lpxL      | LPS       | 8 (36.36)       | 19 (79.17)      | 8.674      | 0.003    |
| ABZJ_00085| Capsule   | 7 (31.82)       | 16 (66.67)      | 5.576      | 0.018    |
| AB57_0990 | Heme utilization | 11 (50)       | 19 (79.17)      | 4.305      | 0.038    |

Abbreviations: VFs, Virulence factors; CRAB, Carbapenem-resistant *Acinetobacter baumannii*; VAP, Ventilator associated pneumonia; CRAB-C, Positive genes of patients with CRAB colonization but without VAP; CRAB-I, Positive genes of CRAB VAP patients.

Discussion

CRAB has a high clinical prevalence and is a common pathogen in VAP, but a positive ETA culture alone cannot effectively distinguish between bacterial colonisation and infection, representing a longstanding clinical challenge in the management of severely ill patients. Therefore, we investigated the difference in LRT microecology between infected and colonised patients using multi-genomics methods, with the goal of clarifying the clinical management of CRAB infection.

In recent years, several studies have shown that changes in the LRT microbiome are related to the occurrence of lung diseases, but few studies have examined the relationship between respiratory microbiota and infection, and most of those focused on pulmonary tuberculosis and
pulmonary fibrosis [13, 43]. In this study, the 16S rRNA analysis of 52 patients revealed that the α and β diversity of the LRT microbiome was significantly lower in CRAB-I patients than in CRAB-C and CRAB-N patients (Fig. 2). The ETA microbiota in the CRAB-N group consisted mainly of Proteobacteria and Haemophilus, consistent with a previous report [44], and was more diverse than in the CRAB-C and CRAB-I groups. The microbiota of the latter two groups were mainly Proteobacteria and Acinetobacter, and the abundance of Acinetobacter in CRAB-I was as high as 76.19% (Fig. 3). Further LEfSe analysis (Fig. 3H) revealed that, in comparison with CRAB-C patients (who were enriched in unidentified Corynebacteriaceae, Nesseria, Nordella, and Streptococcus), CRAB-I patients had a higher abundance of Acinetobacter. The relative abundance of Acinetobacter increased in the order CRAB-N, CRAB-C, and CRAB-I; this trend was confirmed by Woo et al. [45]. Together, these results indicated that a dynamic evolution of pulmonary microbiota, including a decline in diversity and enrichment of Acinetobacter, occurs prior to the onset of CRAB VAP [46-47].

Network analysis revealed that the connections between bacteria were most abundant in CRAB-N, less abundant in CRAB-C, and least abundant in CRAB-I; in parallel, the number of genera negatively associated with Acinetobacter also decreased (6, 5, and 4 negative connections in CRAB-N, CRAB-C, and CRAB-I, respectively). In CRAB-I, only four genera (Klebsiella, Pseudomonas, unidentified Erysipelotrichaceae, and Oscillibacter) were negatively correlated with Acinetobacter (Fig. 4). Zakharkina et al. [46] found that Acinetobacter, Pseudomonas, Staphylococcus, and Burkholderia were negatively correlated with the development of VAP; Wouter et al. [48] found that an increase in the abundance of Lactobacillus and Rothia strains was negatively correlated with the specific microbial infection of VAP patients. These findings suggest that disturbance of the respiratory microbiota relieves negative inhibition of CRAB and is therefore likely to promote infection of the host. However, this idea requires further validation.

Functional metagenomic studies of the respiratory tract microbiome are also valuable for detecting bacterial pathogenesis. Mice infected with Streptococcus pneumoniae and Haemophilus influenzae could cause pulmonary inflammatory responses by activating the MAPK signal pathway [49]. In this study, KEGG functional analysis revealed that genes involved in 40 and 45 metabolic pathways (including oxidative phosphorylation, phenylalanine metabolism, fatty acid degradation) were more abundant in the CRAB-C and CRAB-I groups, respectively, than in the CRAB-N group; moreover, signalling protein pathways were more active in CRAB-C patients than in CRAB-I patients (Fig. 5). A previous review described how assessment of microbial function using metagenomics, metatranscriptomics, and metabolomics has identified metabolites produced by respiratory microbiota (especially fatty acids, sugars, and amino acids) that can influence host immunity [15]. This also indicated that the change in bacterial pathogenicity from CRAB-N to CRAB-I may be associated with more active metabolism; this possibility is worthy of further study.

During progression from colonisation to infection, bacterial invasiveness and toxicity play a key role. Metagenomics analysis revealed that four major virulence gene clusters (iron uptake, siderophore, immune evasion, and biofilm formation) increased in abundance from the CRAB-N to the
CRAB-I group (Fig. 6A). The number of virulence genes annotated and networks constructed was significantly higher in the CRAB-I group in the CRAB-C and CRAB-N groups (Fig. 6C). Wilcoxon tests showed that the abundance of virulence genes related to heme utilisation, such as AB57_0984, AB57_0990, AB57_0992, and mymA, was higher in the CRAB-I group than in the CRAB-C group (Table S4 and Fig. 6B). AB57_0984, a LysR family transcription regulator, is linked to elevated invasiveness [50]. AB57_0990 (a member of the TonB family) and the TonB system play key roles in the pathogenicity of AB [51].

Network diagram and fitted curve analysis confirmed that these virulence genes were associated with Acinetobacter (Fig. 6D and Fig. 7). In addition, WGS of strains from CRAB-C and CRAB-I patients revealed that more virulence genes such as Lpxl, ABZJ_00085, and AB57_0990 were present in the infection group (Table 1 and Fig. 8). The enrichment in virulence of CRAB indicated that enhancement of bacterial pathogenicity could be another key factor that promotes infection after perturbation of the microbiota.

In terms of patient clinical characteristics, we observed no significant differences in gender, age, or severity of disease at the time of enrolment between the three groups. The number of days of mechanical ventilation before collection was smaller in CRAB-N patients than in CRAB-positive patients, and mortality was higher in CRAB-I patients (Table S2). ST208 was the main type (30.43%), followed by ST938 and ST195, and bacterial MLST distribution did not differ significantly between the colonisation and infection group (Fig. 8). All isolates were highly drug-resistant, and $\text{bla}_{\text{oxa-23}}$ was the major determinant of resistance [52].

Our results indicate that to draw conclusions about the importance of microbiota evolution, it will be necessary to perform consecutive observations of individual patients, spanning the period from colonisation to infection. In future studies, transcriptome and proteome analysis could be used to explore germ–host interactions and pathogenesis.

**Conclusions**

By combining 16S rRNA amplicon analysis, metagenomics sequencing, and WGS, we characterised the respiratory tract microbiome of patients with CRAB VAP and explored the differences in microbiota between bacterial colonisation and infection. Our results revealed that LRT microbiome dysbiosis, including declining diversity, the rise of Acinetobacter to dominance, weakening of the negative regulation of Acinetobacter, and significant enhancement of virulence, could promote the occurrence of infection. Thus, multi-genomics investigational methods could be used to develop new diagnostic measures for CRAB VAP.

**Abbreviations**

CRAB: Carbapenem-resistant Acinetobacter baumannii; LRT: lower respiratory tract; VAP: Ventilator associated pneumonia; CRAB-N: LRT microbiota of patients with neither VAP nor CRAB LRT colonization;
CRAB-C: LRT microbiota of patients with CRAB colonization but without VAP; CRAB-I: LRT microbiota of patients who developed CRAB VAP; LefSe: Linear discriminant analysis effect size; LDA: Linear discriminant analysis; DMNC: Density of Maximum Neighborhood Component; VFs: Virulence factors.

Declarations

Ethics approval and consent to participate

Approval was obtained from the ethical board of the Research Ethics Committee of the First Affiliated Hospital of Medicine, Zhejiang University in China (No. 2016-458-1).

Consent for publication

Not applicable.

Availability of data and material

The data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under Bio Project number PRJNA681291 and PRJNA679997. The datasets supporting the conclusions of this article are included within the article and its additional files.

Conflicts of interests

All authors declare no competing interests.

Funding

This study was partially supported by grants from the National Key Research and Development Program of China (2017YFC1200203 and 2017YFC1200205), National Natural Science Foundation of China (81971984 and 31671366).

Authors’ contributions

YHX, HQZ, and TTX designed the study. QG, TTX, ML, LHG, XY, YLL, and CHW, analyzed and interpreted the data. YZZ, YW, STZ, XHC, and XYK collected the samples. PS, KZ, BWZ, QXL, and YBC oversaw the field projects. QF provided clinical support. TTX, QG, and YZZ wrote the manuscript. All authors critically reviewed the manuscript.

Acknowledgments

We thank Jinru Ji, Chaoqun Ying, and Zhiying Liu for providing assistance with bacterial identification, and Dr. Juan Hu for providing support for sample collection.
References

1. Shamsizadeh Z, Nikaeen M, Nasr EB, Mirhoseini SH, Hatamzadeh M, Hassanzadeh A. Detection of antibiotic resistant Acinetobacter baumannii in various hospital environments: potential sources for transmission of Acinetobacter infections. Environ Health Prev Med. 2017;22:44.

2. Papazian L, Klompas M, Luyt CE. Ventilator-associated pneumonia in adults: a narrative review. Intensive Care Med. 2020;46:888–906.

3. Huang Y, Jiao Y, Zhang J, Xu J, Cheng Q, Li Y, et al. Infection Assembly of Shanghai Respiratory Society. Microbial Etiology and Prognostic Factors of Ventilator-associated Pneumonia: A Multicenter Retrospective Study in Shanghai. Clin Infect Dis. 2018;67(suppl_2):146–52.

4. Consales G, Gramigni E, Zamidei L, Bettocchi D, De Gaudio AR. A multidrug-resistant Acinetobacter baumannii outbreak in intensive care unit: antimicrobial and organizational strategies. J Crit Care. 2011;26(5):453–9.

5. Yin Y, Zhao C, Li H, Jin L, Wang Q, Wang R, et al. Clinical and microbiological characteristics of adults with hospital-acquired pneumonia: a 10-year prospective observational study in China. Eur J Clin Microbiol Infect. 2021;Dis 40(4):683–690.

6. Hu FP, Guo Y, Zhu DM, Wang F, Jiang XF, Xu YC, et al. Resistance trends among clinical isolates in China reported from CHINET surveillance of bacterial resistance, 2005–2014. Clin Microbiol Infect. 2016;22(Suppl 1):9–14.

7. Harding CM, Hennon SW, Feldman MF. Uncovering the mechanisms of Acinetobacter baumannii virulence. Nat Rev Microbiol. 2018;16:91–102.

8. Uppalapati SR, Sett A, Pathania R. The Outer Membrane Proteins OmpA, CarO, and OprD of Acinetobacter baumannii Confer a Two-Pronged Defense in Facilitating Its Success as a Potent Human Pathogen. Front Microbiol. 2020;11:589234.

9. Xie J, Yang Y, Huang Y, Kang Y, Xu Y, Ma X, et al. The Current Epidemiological Landscape of Ventilator-associated Pneumonia in the Intensive Care Unit: A Multicenter Prospective Observational Study in China. Clin Infect Dis. 2018;67(suppl_2):153–61.

10. WHO Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed. Available online: https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (accessed on 01 August 2020).

11. Dickson RP, Erb-Downward JR, Huffnagle GB. Towards an ecology of the lung: new conceptual models of pulmonary microbiology and pneumonia pathogenesis. Lancet Respir Med. 2014;2:238–46.

12. Kelly BJ, Imai I, Bittinger K, Laughlin A, Fuchs BD, Bushman FD, et al. Composition and dynamics of the respiratory tract microbiome in intubated patients. Microbiome. 2016;4:7.

13. Emonet S, Lazarevic V, Leemann Refondini C, Gaïa N, Leo S, Girard M, et al. Identification of respiratory microbiota markers in ventilator-associated pneumonia. Intensive Care Med. 2019;45(8):1082–92.
14. Faner R, Sibila O, Agustí A, Bernasconi E, Chalmers JD, Huffnagle GB, et al. The microbiome in respiratory medicine: current challenges and future perspectives. Eur Respir J. 2017;49(4):1602086.
15. Budden KF, Shukla SD, Rehman SF, Bowerman KL, Keely S, Hugenholtz P, et al. Functional effects of the microbiota in chronic respiratory disease. Lancet Respir Med. 2019;7(10):907–20.
16. Hakansson AP, Orihuela CJ, Bogaert D. Bacterial-Host Interactions: Physiology and Pathophysiology of Respiratory Infection. Physiol Rev. 2018;98:781–811.
17. Roquilly A, Torres A, Villadangos JA, Netea MG, Dickson R, Becher B, et al. Pathophysiological role of respiratory dysbiosis in hospital-acquired pneumonia. Lancet Respir Med. 2019;7(8):710–20.
18. Horan TC, Andrus M, Dudeck MA. CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. Am J Infect Control. 2008;36:309–32.
19. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. CLSI Approved Standard M100-S15. Wayne: Clinical and Laboratory Standards Institute; 2018.
20. European Committee on Antimicrobial Susceptibility testing (EUCAST). Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST polymyxin breakpoints working group. 2016. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf.
21. LeGall JR, Loirat P, Alpérovitch A. APACHE II—a severity of disease classification system. Crit Care Med. 1986;14:754–5.
22. Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011;27:2957–63.
23. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods. 2013;10(1):57–9.
24. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335–6.
25. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27:2194–200.
26. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:D590-6.
27. Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 2004;5:113.
28. Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. metaSPAdes: a new versatile metagenomic assembler. Genome Res. 2017;27:824–34.
29. Menzel P, Ng KL, Krogh A. Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nat Commun. 2016;7:11257.
30. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2015;12(1):59–60.

31. Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 2017;45:D353–61.

32. Chen LH, Zheng D, Liu B, Yang J, Jin Q. VFDB 2016: hierarchical and refined dataset for big data analysis–10 years on. Nucleic Acids Res. 2016;44:D694-7.

33. Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Zaslavsky L, et al. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 2016;44(14):6614–24.

34. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, et al. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics. 2015;31(22):3691–3.

35. Price MN, Dehal PS, Arkin AP. FastTree 2–approximately maximum-likelihood trees for large alignments. PLoS One. 2010;5:e9490.

36. Jain C, Rodriguez RLM, Phillippy AM, Konstantinidis KT, Aluru S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nat Commun. 2018;9:5114.

37. Page AJ, Taylor B, Softw JKJOS. Multilocus sequence typing by blast from de novo assemblies against PubMLST. theoj.org. 2016.

38. Hall BG. Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol. 2013;30:1229–35.

39. Seemann T. Abricate. Available from: https://github.com/tseemann/abricate. Accessed: 11th February, 2018.

40. Wyres KL, Cahill SM, Holt KE, Hall RM, Kenyon JJ. Identification of Acinetobacter baumannii loci for capsular polysaccharide (KL) and lipooligosaccharide outer core (OCL) synthesis in genome assemblies using curated reference databases compatible with Kaptive. Microb Genom. 2020;6(3):e000339.

41. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60.

42. Csardi G, Nepusz T. The Igraph software package for complex network research. Inter J Complex Syst. 2006;1695:1–9.

43. Krishna P, Jain A, Bisen PS. Microbiome diversity in the sputum of patients with pulmonary tuberculosis. Eur J Clin Microbiol Infect Dis. 2016;35:1205–10.

44. Dickson RP, Erb-Downward JR, Freeman CM, McCloskey L, Falkowski NR, Huffnagle GB, et al. Bacterial Topography of the Healthy Human Lower Respiratory Tract MBio. 2017;8(1):e02287-16.

45. Woo S, Park SY, Kim Y, Jeon JP, Lee JJ, Hong JY. The Dynamics of Respiratory Microbiota during Mechanical Ventilation in Patients with Pneumonia. J Clin Med. 2020;9(3):638.

46. Zakharkina T, Martin-Loeches I, Matamoros S, Povoa P, Torres A, Kastelijn JB, et al. The dynamics of the pulmonary microbiome during mechanical ventilation in the intensive care unit and the association with occurrence of pneumonia. Thorax. 2017;72(9):803–10.
47. Dickson RP, Singer BH, Newstead MW, Falkowski NR, Erb-Downward JR, Standiford TJ, et al. Enrichment of the lung microbiome with gut bacteria in sepsis and the acute respiratory distress syndrome. Nat Microbiol. 2016;1(10):16113.

48. de Steenhuijsen Piters WA, Huijskens EG, Wyllie AL, Biesbroek G, van den Bergh MR, Veenhoven RH, et al. Dysbiosis of upper respiratory tract microbiota in elderly pneumonia patients. ISME J. 2016;10(1):97–108.

49. Weiser JN, Ferreira DM, Paton JC. Streptococcus pneumoniae: transmission, colonization and invasion. Nat Rev Microbiol. 2018;16:355–67.

50. Gebhardt MJ, Czyz DM, Singh S, Zurawski DV, Becker L, Shuman HA. GigC, a LysR Family Transcription Regulator, Is Required for Cysteine Metabolism and Virulence in Acinetobacter baumannii. Infect Immun. 2020;89(1):e00180-20.

51. Runci F, Gentile V, Frangipani E, Rampioni G, Leoni L, Lucidi M, et al. Contribution of Active Iron Uptake to Acinetobacter baumannii Pathogenicity. Infect Immun. 2019;87(4):e00755-18.

52. Fu Y, Zhou J, Zhou H, Yang Q, Wei Z, Yu Y, et al. Wide dissemination of OXA-23-producing carbapenem-resistant Acinetobacter baumannii clonal complex 22 in multiple cities of China. J Antimicrob Chemother. 2010;65(4):644–50.

Figures
Figure 1

Flowchart of the patient enrollment process.
Figure 2

Phylogenetic diversity comparison of LRT microbiota in CRAB-N, CRAB-C and CRAB-I patients. (A) Box plots depict microbiota diversity differences according to the Shannon index among three group. (B) Box plots depict microbiota diversity differences according to the Gini Simpson index among three group. The upper and lower ranges of the box represent the 75% and 25% quartiles, respectively. (C) The x-axis shows PCo 1 and the y-axis PCo 2 form Bray-Curtis distances for β-diversity. The changes per patient are visualized in as dashed lines dark red for CRAB-I, in blue for CRAB-C and in gray for CRAB-N. Logistic regression analysis showed that the change of PCo1 was significantly lower in patients who developed pneumonia (P=0.001). (D) The x-axis shows MDS 1 and the y-axis MDS 2 form Bray-Curtis distances for β-diversity (Stress=0.113). The changes per patient are visualized in as dashed lines dark red for CRAB-I, in blue for CRAB-C and in gray for CRAB-N. Significant differences are indicated by *P<0.05, **P <0.01, ***P <0.001, ****P <0.0001.
Figure 3

Comparison of phylum and genus of the LRT microbiota in the three patient groups and Linear discriminant analysis effect size (LEfSe) analysis of the microbiota at genus level. (A) Comparison of the average abundance of each bacterial phylum in each group, respectively. (B) Comparison of the average abundance of bacterial phylum in each patient, respectively. (C) Comparison of the average abundance of each bacterial genus in each group, respectively. (D) Comparison of the average abundance of
bacterial genus in each patient, respectively. (E) LDA scores indicated significant differences in the microbiota among the CRAB-N (gray), CRAB-C (blue) and CRAB-I patients (red). (F) LDA scores indicated significant differences in the microbiota between the CRAB-N (gray) and CRAB-C patients (blue). (G) LDA scores indicate significant differences in the microbiota between the CRAB-I patients (red) and CRAB-N controls (gray). (H) LDA scores indicate significant differences in the microbiota between the CRAB-I (red) and CRAB-C patients (blue). LDA scores > 3.5. The subject group is indicated by the color key at the top right corner.
Figure 4

Co-occurring network of microbial communities in LRT samples from CRAB-N, CRAB-C and CRAB-I patients. Note: A co-occurring network containing strong ($\rho > 0.6$) and significant (FDR-adjusted $P < 0.05$) correlations was represented. Each node represents a genus and the nodes are colored by phylum. The size of each node is proportional to the number of connections. The thickness of each edge is proportional to the $\rho$. Light blue lines represent negative correlations, and red lines represent positive correlations.

Figure 5

Functionally distinct and Linear discriminant analysis effect size (LEfSe) analysis based on KEGG pathway. (A) Linear discriminant analysis (LDA) scores indicate significant differences in the microbiota between the CRAB-C patients (blue) and CRAB-N controls (gray); (B) LDA scores indicate significant differences in the microbiota between the CRAB-I patients (red) and CRAB-N controls (gray); (C) LDA scores indicate significant differences in the microbiota between the CRAB-C patients (blue) and CRAB-I patients (red). LDA scores $> 2.5$. Abbreviations: CRAB, Carbapenem-resistant Acinetobacter baumannii;
LRT, Lower respiratory tract; VAP, Ventilator associated pneumonia; CRAB-N, LRT microbiota of patients with neither VAP nor CRAB LRT colonization; CRAB-C, LRT microbiota of patients with CRAB colonization but without VAP; CRAB-I, LRT microbiota of patients who developed CRAB VAP.

**Figure 6**

Comparison of virulence factors and Network analysis. (A) Summary of the top 10 relative abundances of function group of virulence genomes in each group; (B) LEfSe and LDA scores indicated significant differences in the virulence genes among the CRAB-N (gray), CRAB-C (blue) and CRAB-I patients (red). (C) Co-occurring network of virulence genomes communities in LRT samples from three patient groups. The nodes are colored by the function of virulence genomes, the upper right corner shows DMNC; (D) Co-occurring network of virulence genomes and microbiota communities in LRT samples from three patient groups based on correlation analysis. The nodes are shaped by virulence genomes and bacteria, sized by the relative abundances and colored by the function of virulence genomes or phylum. The connections in
the network represent a strong ($r > 0.8$) and significant ($q < 0.05$) correlations. The size of each node is proportional to the number of connections. Light blue lines represent positive correlations, and red lines represent negative correlations.

**Figure 7**

Correlation between the sum of the relative abundances of genus or species and the significant virulence genes density. (A) Correlation between the sum of the relative abundances of Acinetobacter and Methylorubrum and the significant virulence genes density in each sample ($R^2 = 0.529, p < 0.0001$; Pearson's correlation). (B) Correlation between the sum of the relative abundances of species and the significant virulence genes density in each sample ($R^2 = 0.755, p < 0.0001$; Pearson's correlation). Abbreviations: $V_{abun}$, Sum of abundance of significant virulence genes; ${{BA}_i}$: abundance of bacteria $i$. 

$$V_{abun} = 0.013798 \times BA_{Acinetobacter} + 10.493319 \times BA_{Methylorubrum} + 0.003546$$
Figure 8

Molecular type, resistant and virulence genes analysis of Acinetobacter baumannii isolates.

Supplementary Files

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