Airway microbiome research: a modern perspective on surveillance cultures?

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Abstract: The incidence of ventilator-associated pneumonia (VAP) is estimated to be around 10% in a high-risk population. Over the last decade, major improvements have been made in the prevention of VAP, with great cost-effectiveness. However, we still do not understand the exact pathogenesis of VAP. A better understanding might explain why some patients develop ventilator-associated tracheobronchitis, while others develop VAP even though they are infected with the same types of pathogens. Microbiome research has been a hot topic in translational medicine over the past decade. Slowly, microbiome research has also been introduced to the intensive care setting. One of the areas where it may influence our pathophysiological considerations is in VAP. The adapted island has been proposed for the colonization and infection of the respiratory tract. In this model, not only the immigration of bacteria into the lung is important, but elimination and regional growth factors are of equal significance. The importance of these factors can be supported by epidemiological studies. Several small observational studies on the development of the pulmonary microbiome during mechanical ventilation also support this theory. We speculate on the consequences of the newest insights in microbiome research on the prevention and targeted treatment of VAP. We conclude that there is still a strong need for more in-depth analyses of the changes in the microbial composition of the pulmonary microbiome during mechanical ventilation and with the development of VAP.

Keywords: Ventilator-associated pneumonia (VAP); microbiome; prevention

Ventilator-associated pneumonia (VAP)

The incidence of VAP is estimated to be around 10% in a high risk population of critically ill patients who are mechanically ventilated for more than 48 h (1). It is unclear if VAP is responsible for an increase in mortality (2), but it is strongly associated with an increase in ICU-length of stay, morbidity and healthcare costs (1,3). The optimal strategy to decrease the impact of VAP is three-folded: (I) prevent when possible; (II) early and targeted treatment when pneumonia develops; and (III) withholding antibiotics from those patients that develop symptoms but are not infected.

Over the last decade, major improvements have been made in the prevention of VAP, with great cost-effectiveness (4). Preventive measures have been targeted against the two pathophysiological principles of VAP development; micro-aspiration of bacteria that accumulate above the endotracheal tube (oral hygiene, semi-recumbent position, cuff pressure and suctioning of above the cuff) and the introduction of pathogens from the environment (hand hygiene, bacterial filters and minimal manipulation of the tubing). These pathophysiological principles have been relatively unchallenged over the last decades
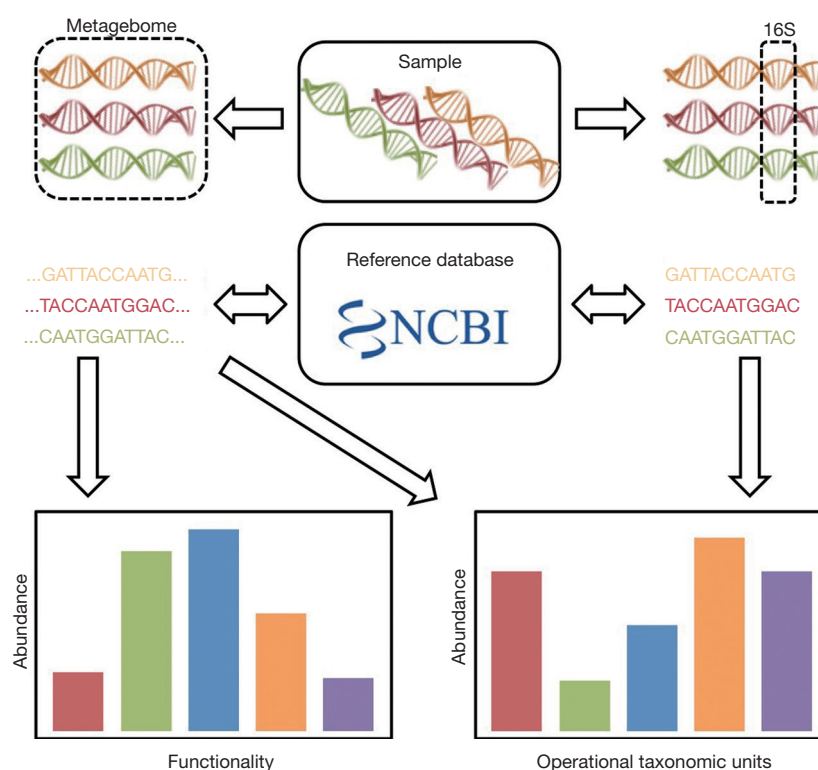
Figure 1 16S rRNA phylogenetic sequencing and metagenomic sequencing

but fail to explain why patients who develop VAP and ventilator-associated trachea-bronchitis (VAT) carry the same pathogens (1). This suggests that there are either undiscovered pathogen factors that influence pathogenicity or that there are host or environmental factors that influence the development of VAP.

In this review, we will discuss recent advances in molecular biology, which allow for the culture-independent evaluation of the bacterial flora in the human body. Microbiome research in intubated and mechanically ventilated patients is in its infancy but has already shed light on several factors in the pathophysiology of VAP, which will be described in this review. A deepened understanding of the pathophysiology of VAP allows for better prevention and treatment. Furthermore, culture-independent techniques might allow for more targeted treatment of the causative pathogen in the near future.

Microbiome research

Microbiome research has been made possible through the development of high-throughput sequencing. Different types of analysis can be performed, depending on the deepness of nucleic acid sequencing. In general, there are two types of sequencing procedures (5). Metagenomics relies on the sequencing of all nucleic acids sequences obtained in a sample. This technique allows for full genome sequence, for instance of new species in a sample. In contrast, analysis of bacterial microbiota can also be performed based on certain conserved regions in the genome, which allows for the generation of a phylogenetic tree, which described the proportion of each bacterial species or phyla in a sample (*Figure 1*).

Phylogenetic sequences

Microbiota analysis is based on sequencing of specific regions of the DNA genes encoding the 16S ribosomal RNA (rRNA) subunit (6). This is made possible by the presence of regions that are conserved throughout the bacterial kingdom within these genes, which allows PCR amplification using universal primers. Eight hypervariable regions with sequences specific for each bacterial phyla and species separate these conserved regions. After next

generation sequencing of the PCR amplified hypervariable regions, the sequence alignment on pre-identified sequence libraries permits the determination of bacterial composition in samples.

Strengths and limitations

A great advantage of sequencing techniques over conventional microbiological culture is its capability to identify hard-to-grow or even uncultivable bacteria. The 16S rRNA sequencing is also superior over conventional microbiology in terms of the possibility for sample storage as the DNA is stable when frozen, while culture dependent techniques rely on living bacteria and therefore fresh material. Several limitations should be understood in order to correctly interpret microbiota data. First, interpretation of 16S rRNA sequencing data relies on the number of hypervariable regions amplified and sequenced. Indeed, the precision of bacterial composition of a sample will vary depending on this number. In addition, the number of sequences obtained from a sample will determine the deepness of microbiota analysis. On the one hand, the greater hypervariable regions are sequenced and the greater number of sequences is obtained for a sample, the greater details are provided in term of bacterial species composition. On the other hand, the full length of 16S rRNA genes does not allow its complete sequencing for microbiota study in routine. Also, in order to reduce cost, many samples may be sequenced in the same run, reducing the number of sequenced obtained for each sample. Thus, a compromise has to be done between number of hypervariable regions, number of samples sequenced in the procedure, and financial and technical possibilities allowed for the study. Most recent studies also select one or two hypervariable regions and use the variation in those to generate phylogenetic trees. Based on these 16S rRNA sequences, the separation of bacterial species are poorly effective within some phyla, such as the *Streptococcus* (7).

Second, in contrast to conventional microbiology, microbiota analysis provides only proportional abundance of bacterial phyla and species and identifies both dead and live bacteria. Due to these characteristics, phylogenetic sequences do not allow for a quantitative measurement of the number of copies of specific bacterial species. This has to be integrated for data interpretation when repeated samples in a short period of time are performed (8,9).

Specificities for the lung

The lungs have a complex architectural structure from the trachea to the alveoli. Therefore, microbiota may not be homogenous throughout the lungs and this make interpretation of data somewhat difficult to compare. Various methods are available to obtain samples of the bacterial population of the airways. In ICU patients, the two most available technics are tracheal aspirations and bronchoalveolar lavages. However, the sampling methods by themselves may induce a contamination from proximal parts such as the oropharyngeal or oronasal cavity, or the tracheal tube for ventilated patients. A parallel sampling and sequencing of these anatomical parts may permit identification of bacterial contamination and of specific bacterial population in deeper samples.

Pathophysiology

Microbiome research has shown that the lungs of healthy subjects are not sterile (10), in contrast to conventional wisdom of less than a decade ago (11). The existence of distinct microbial communities within the body changes the perspective for many inflammatory and infectious diseases (10). Much research on microbial communities in disease has been performed in the gut (12). There are two important differences between the gut and the lung that result in caution when translating findings from gut microbiome research to the respiratory system. First, the bacterial load in the gut is extremely high under healthy circumstances ($\sim10^8$ copies/mL), while the bacterial load in the lung is extremely low as compared to the rest of body exterior ($\sim10^4$ copies/mL) (13). Furthermore, it is uncertain if the bacteria in a healthy lung do reproduce or are just "passing by" (11). Thus, under normal circumstances the lung has no resident microbiome that is resistant to invading microbes. Second, the gut only allows unidirectional travel by design, while all conducting airways in mammals require two-way traffic. Not only all microbes enter the lung through the mouth, must they exit that same way. Oral flora is abundant and diverse and is the healthy microbiome of the lower respiratory tract is closely related to the oral microbiome under normal circumstances (13,14).

Whiteson *et al*. further developed these concepts through the comparison of the oral cavity to a mainland and the lungs as island in front of the coast (15). They applied "island bibliography" to describe the diversity of the microbial community in the oral cavity and the lung. In patients with cystic fibrosis this model seemed to describe the results quite accurately (16,17). Dickson *et al*. further developed this idea into the "adapted island model". In a hallmark
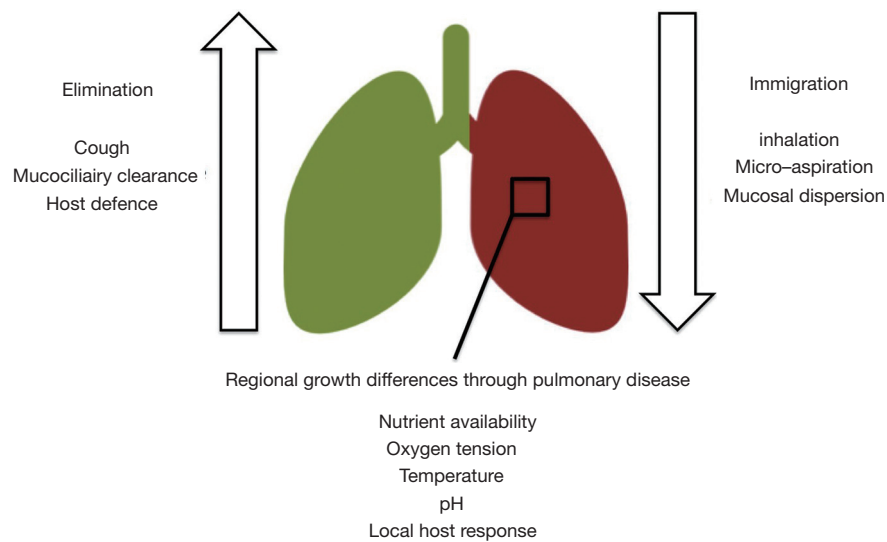
Figure 2 Adapted island model. Personal interpretation of previously published figures, see for example (18).

paper, they describe three contributing factors to a change in the pulmonary microbiome: immigration, elimination and regional growth conditions (*Figure 2*).

The development of VAP is generally described as an increased immigration through inhalation or micro-aspiration of pathogens into the lung. This process is facilitated through the endotracheal tube. Indeed, two studies showed a decrease in bacterial diversity in the pulmonary microbiome with prolonged mechanical ventilation (19,20). There was no association between the degree to which the diversity decreased and the development of VAP. However, in patients who developed VAP *Pseudomonadales* (including *Pseudomonas* species and *Acinetobacter* species) increased to a greater extent than in patients that did not develop VAP. This was irrespective of the development of colonization in the control group, which suggests that an increase in opportunistic bacteria such as *Pseudomonas* is associated with VAP, even if another pathogen is causing the pneumonia (19).

Elimination is also hampered in intubated and mechanically ventilated patients. Positive pressure ventilation, sedation and neuro-muscular blockage hamper the possibility to effectively cough up sputum and thereby eliminate bacteria. Positive pressure ventilation also hampers mucociliary clearance and the innate immune response is frequently disturbed in critically ill ICU-patients (21). Indeed, one study comparing patients that developed VAT to those that developed VAP found that the complement response was severely hampered in those

patients progressing to pneumonia (22). This might also put the higher risk of VAP of neurosurgical patients into a perspective as they frequently have a hampered cough response and altered immune response (23).

Regional growth conditions in the lung can be the consequence of any condition that affects the lung heterogeneously. Intubation and mechanical ventilation frequently results in atelectasis of the dependent lung regions, which may put a selective pressure on specific bacteria. This might explain why a ventilation strategy with a higher positive end-expiratory pressure decreases the risk of VAP (24). Acute respiratory distress syndrome (ARDS) is characterized by protein rich pulmonary edema, which contain high level of amino acids, products of glycolysis and lipids that may form a substrate for bacterial metabolism (25-27). Empiric clinical evidence supports these experimental findings as patients with ARDS are at greater risk for VAP (28). Community-acquired pneumonia is another risk factor for VAP that may be explained through regional growth differences; the infected part of the lung is damaged and collapsed, resulting in regional conditions that may favor one pathogen. Finally, chronic pulmonary diseases such as COPD are associated with an altered respiratory microbiome in spontaneously breathing patients and it may play a role in the development of exacerbations (29-31). Alterations in the pulmonary microbiome and increased microbial loads might also be a cause for dysbiosis induced VAP; pneumonia developing by selective pressure on the existing microbiome towards the selection of a

single bacterial species that continues to cause pneumonia but there is currently no evidence for this statement in ventilated patients.

Implications for prevention

Prevention of VAP mainly focuses on the prevention of immigration of bacteria into the lung. Some of the interventions that are proven to prevent VAP are; minimize sedation, supine positioning, oral hygiene measurements, maintenance of the ventilator circuit, decontamination of the mouth or gut, continuous control of cuff pressure and subglottic suctioning (32). Prevention of immigration of bacteria into the lung should, in theory, prevent colonization and VAT as well as VAP. However, these measures will not reduce the incidence of VAP in those patients in whom the lower airways do get colonized with a high number of bacteria.

Increased elimination has been tested as a prevention measure through the administration of systemic or inhaled antibiotics for the prevention of VAP. The evidence for pre-emptive treatment of patients at high risk for VAP is promising, but inconclusive until now (33-35). The available small randomized controlled trials suggest a reduction in VAP incidence with both systemic and inhaled antibiotics, but the effect on total antibiotic consumptions is unknown. A recent observational cohort study comparing selective decontamination with and without systemic antibiotic therapy suggests that a short course of cephalosporine therapy does decrease the incidence of VAP, without increasing antibiotic exposure (36). Other methods to increase elimination is through cough support or a lateral Trendelenburg position of the patient (37).

The potential of a surveillance based therapy strategy

Assuming that all pathogens that cause VAP immigrate into the lung during mechanical ventilation, they will have to pass the trachea at all times. Longitudinal studies with daily cultures of endotracheal aspirates suggest that in the majority of the patients the trachea is colonized with causative pathogen at least 1–2 days before pneumonia develops (38,39). However, colonization does frequently not progress to pneumonia. A treatment approach based on previous surveillance culture results was proposed, but was outperformed by a empirical, guideline based strategy (40).

This was mainly attributed to the fact that only the results of cultures taken 3–7 days before the start antibiotic therapy were available at the time of treatment decision (40). Culture-independent detection of bacteria in endotracheal aspirates might provide faster results and thus might revive the possibility of surveillance based antibiotic therapy. The results of 16S based phylogenetic sequencing do not compare well to the results of cultures in sputum (19,20) and are therefore not useful for this purpose. More targeted assays might allow for a quantitative analysis of the number of gene copies per micro-organism (41). The major challenge with assessment of genetic material alone is that this does not indicate if it is a reproducing organism or not. Therefore, a combination of genetics with a metabolic product of the bacteria or the host immune response might be more suitable. Indeed, there is evidence suggesting that cytokines in bronchoalveolar lavage fluid or bacterial metabolites in exhaled breath can be used to diagnose VAP (42-45). The shift from culture based surveillance to a molecular assay indicative for the development of VAP could also help in earlier treatment, which might allow for a shorter course of systemic antibiotics or even the use of inhaled antibiotics alone. If the diagnostic test were to be sufficient sensitive and specific, this would save the use of longer courses of empiric, broad-spectrum antibiotics in patients suspected of VAP.

Conclusions

Microbiome research has been a hot topic in translational medicine over the past decade. Slowly, microbiome research has also been introduced to the intensive care setting. One of the areas where it may influence our pathophysiological considerations is in VAP. Many hypotheses can be made based on the adapted island model and some of these have already been tested. There is a strong need for more in-depth analyses of the changes in the microbial composition of the pulmonary microbiome during mechanical ventilation and with the development of VAP.

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Footnote

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