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Revealing the dynamics of polymicrobial infections: implications for antibiotic therapy

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Abstract

As a new generation of culture-independent analytical strategies emerge, the amount of data on polymicrobial infections will increase dramatically. For these data to inform clinical thinking, and in turn to maximise benefits for patients, an appropriate framework for their interpretation is required. Here, we use cystic fibrosis (CF) lower airway infections as a model system to examine how conceptual and technological advances can address two clinical questions that are central to improved management of CF respiratory disease. Firstly, can markers of the microbial community be identified that predict a change in infection dynamics and clinical outcomes? Secondly, can these new strategies directly characterize the impact of antimicrobial therapies, allowing treatment efficacy to be both assessed and optimized?

Culture-independent analysis of polymicrobial infection

The assessment of bacterial infections has recently been facilitated by the use of culture-independent tools. These strategies have led to fresh insights into the often complex and dynamic relationships between host and microbes. One field for which recent findings highlight the importance of defining these complex relationships is that of chronic polymicrobial infections: these respond very differently to antibiotic treatment than predicted by conventional models of infections [1-6]. Further, evidence is emerging that the manner by which antibiotics provide clinical benefit in chronic polymicrobial infections might differ from the traditionally accepted mechanisms [7]. Whilst presenting opportunities for the development of novel therapeutic strategies [8,9], these new findings also necessitate a fundamental re-evaluation of the way in which the impact of antimicrobial treatment is assessed *in vivo*.

A new generation of molecular profiling techniques is emerging that greatly increases our ability to analyze and understand complex microbial communities. Consequently, the amount of data derived using these analytical strategies will soon expand dramatically [10]. For these data to inform clinical thinking, and in turn result in maximum benefit to patients, an appropriate framework for their interpretation is required. To date, most molecular profiling-based studies of polymicrobial infections have characterized diversity, and in doing so identified species not routinely isolated by culture-based diagnostics. However, the present challenge is to determine how infections develop and respond to treatment, and this requires the iterative analysis of the complex and dynamic interactions that occur between patient and microbes.

This review will focus on how these new approaches are being used to address three questions that are key to improving the management of infection. First, can we use these diagnostic strategies to define further the microbes present in these complex infections? Second, can these new strategies directly characterize the longitudinal impact of antimicrobial therapies to allow treatment efficacy to be both assessed and optimized? Third, can microbial markers be identified that are predictive of a change in infection status, clinical outcomes, or response to therapy? Answering these questions will require the development of an integrated system for providing clinicians with complex molecular profiling data in a way that can inform the design of treatment strategies. This review provides a framework for using next-generation molecular profiling techniques to deliver translatable clinical benefits for polymicrobial infections using cystic fibrosis (CF) respiratory disease as an example.

Analyzing chronic and complex infections

Koch's postulates [11] established a basis for defining the microbial causation of an infectious disease and provided a basis for modern diagnostic microbiology. Here, specific growth strategies are used to isolate a pure culture of an infectious agent from a clinical sample. Subsequently, determining the impact of an antimicrobial therapy aimed at eradicating any pathogen detected using culture is straightforward; if the patient improves clinically, even after discontinuation of therapy, the infection is deemed contained. Confirmation of this fact can be obtained through the analysis of subsequent samples to see whether the agent is still present. However, culture-based detection of infectious agents does have an important and fundamental weakness: many known clinically important pathogens are difficult to grow *in vitro*, and a significant effort is required for their isolation, and thus detection.

The development of PCR amplification provides a means to detect etiological agents in clinical samples without *in vitro* cultivation. The application of such PCR-based assays effectively removes the need to identify laboratory conditions required for pathogen cultivation, frequently a difficult step. However, it is important to recognize that the use of such culture-independent techniques effectively addresses the same question as traditional culture-based microbiology – can the etiological agent suspected of causing an infection be detected in material recovered from that infection? This diagnostic process, often effective in the detection of known pathogens in acute, single-agent infections, is shown in Figure 1.

One important example in which the determination of the presence or absence of a single infectious agent does not provide sufficient information to allow assessment of treatment efficacy is that of chronic infections. Here, eradication of the infectious agent is typically not realistically achievable. This is the case in the chronic infections of the lower airways of adult CF patients by the Gram-negative bacterium *Pseudomonas aeruginosa*. In circumstances such as these the aim of treatment is not the eradication of the infectious

agent but is instead its management in a way that provides clinical benefit. These treatments are designed to suppress bacterial levels at the site of infection or, in some cases, inhibit the expression of virulence genes [1,7]. Here the ability to determine accurately the number of viable bacterial cells present, and the genes that they are expressing, would provide an essential adjunct to clinical assessment in determining whether a particular intervention has had a positive impact (Figure 1). The recommended use of semi-quantitative culture-based analysis for CF respiratory secretions addresses this to some degree [12], however, the contribution of factors such as the presence of viable but nonculturable bacterial cells [13] means that more reliable and accurate data would be of value. However, such data are not currently readily available to clinicians managing the treatment of CF patients, and clinicians must rely on other sources of information to infer efficacy, as discussed below.

The second context in which single-species microbial analysis is inadequate concerns those infections that involve a complex polymicrobial community. Under these circumstances, different microbes within the community can interact such that the resulting infection pathogenesis ('virulence') differs from that in infections caused by the component species individually [5]. Chronic bacterial infections associated with CF airway disease have been studied by a range of culture-independent profiling methodologies [14], including terminal restriction fragment length polymorphism (T-RFLP) profiling [15,16], 16S ribosomal RNA gene clone sequencing [17], oligonucleotide arrays [18], pyrosequencing [19], and mass-spectrometry-based biosensors [20]. Each approach has revealed greater microbial diversity than was previously recognized. Additional advances using methods that exclude non-viable bacterial cells and extracellular DNA from analysis have also significantly improved the accuracy and specificity of these techniques [21,22]. Overall, the results of these studies suggest that the polymicrobial nature of CF infections could play a key role in driving disease and response to therapy and, in turn, significantly impact upon clinical outcomes [15,23-26].

In short, CF airway disease represents both chronic and polymicrobial infections, and therefore species-specific or culture-based analysis strategies are inadequate for determining treatment efficacy for this condition.

Treatment of CF lung infections

Although the lungs of patients with CF are considered to be normal *in utero*, the process of airway colonization is thought to begin shortly after birth, typically progressing towards chronic infection and inflammation. Once established, lung infections in CF patients are characterized by periods of relative stability punctuated by pulmonary exacerbations [27]. These CF pulmonary exacerbations (CFPE) are episodes of increasing pulmonary symptoms, and present clinically with changes in cough, sputum production, dyspnoea, decreased energy level and appetite, weight loss and decreases in spirometric parameters [27]. The occurrence of CFPE significantly impacts upon both quality of life [28] and survival [29]. During CFPE, antibiotics are administered to reduce sputum bacterial load [30] with an expectation of an improvement in pulmonary symptoms [31].

The direct treatment of CF respiratory disease involves five primary therapeutics: antibiotics, bronchodilators, mucolytic agents, anti-inflammatory drugs, and airway clearance techniques such as autogenic drainage and exercise plus oxygen therapy [32]. Whilst all of these approaches might have direct or indirect impacts on lung infections, we will focus here on the use of antibiotics as the central antimicrobial strategy. Further, as illustrated by the dramatic increase in life expectancy in CF patients following the introduction of antibiotic therapy, this is an approach with the potential to have a major influence on the course of respiratory CF infections [33].

Antibiotics are employed in the treatment of CF in three ways: (i) as a maintenance therapy, (ii) in response to laboratory evidence of new pulmonary infection with a known pathogen, and (iii) to treat infectious exacerbations. Treatments aimed at mitigation of CFPE typically consist of administering two anti-pseudomonal antibiotics, to reduce the development of resistance and provide an additive or synergistic effect on bacterial killing, for approximately 10–14 days [30,34]. It is on this treatment of CFPE that we focus here.

Pulmonary exacerbations are the single most important cause of morbidity in CF [35], the number of exacerbations and courses of intravenous (IV) antibiotics per year is strongly related to decline in lung function [29]. Furthermore, a substantial fraction of CF patients experiencing a pulmonary exacerbation fail to recover pre-exacerbation lung function with standard therapy [36], suggesting that improved treatment of exacerbations could improve long-term outcomes. It is therefore vital for the long-term health of this patient group to ensure that an effective intervention is made to limit as much as possible the resulting damage to the airways. Further, as the life expectancy of CF patients increases, the significance of side effects that result from repeated antibiotic treatments, particularly renal impairment [37], ototoxicity [38], and increased risk of drug reactions [39], becomes even more important. When combined with the importance of minimizing the impact of bacterial resistance to antibiotics, there is a clear need for antibiotics to be administered as efficiently as possible.

Conventionally, the selection of antibiotic treatment is based on estimates of the antimicrobial sensitivities of a limited number of species cultured from recent sputum specimens [35]. In some cases the selection of therapy might have little basis in empirical microbiological data. For example, when faced with ill patients for whom recent culture results are not available, therapy might be chosen presumptively to cover known CF pathogens such as *P. aeruginosa* for which specific antibiotics are particularly effective *in vitro* [34]. Once administered, assessment of the success of treatment is currently performed based on data derived in three ways: clinical signs, patient feedback, and culture-based, diagnostic microbiology. The value of culture results in defining improvement is open to question as diagnostic microbiology is currently focused on the presence or absence of a small number of species regarded as being of key clinical importance [12,27,40] in a context where eradication is unlikely to be achieved. Although quantitative culture can be used to characterize the impact of antibiotic therapy on key pathogens [31], it is not performed routinely. Therefore, effectively, the impact of antimicrobial therapy is judged indirectly, based on changes in clinical signs and patient feedback. It has recently been shown that this combination of methods is not reliable in predicting which CF patients will derive clinical benefit [6,41].

The challenges of determining antibiotic impact

The process of predicting the likely impact of antibiotic therapy, or measuring the impact following treatment, is far more complex than the current approach allows. Many factors contribute to this complexity. First, prediction of the efficacy of a specific treatment needs to take into account a number of issues that are not currently considered. For example, the poor correlation between the results of *in vitro* sensitivity testing of individual bacterial species and subsequent treatment success [6,42,43] probably results, in part, from the fact that the mode of *in vitro* growth of a species often differs from the mode of growth at the site of infection. It is likely that this lack of correlation is also influenced by incomplete sampling of the bacterial diversity within the sample [42,43]. Further, in CF, several factors are likely to reduce the susceptibility of bacteria to antibiotics, including the growth of many of the bacterial species commonly isolated as biofilms [3,44,45], the anaerobic nature of mucus plugs, and the inhibition of certain groups of antibiotics by components of purulent sputum

[46]. A number of other key issues must be also be considered when attempting to predict the likely antimicrobial impact of therapy, including: (i) the relationship between mode of drug delivery, likely airway penetration, and the physical distribution of bacteria, (ii) the phase or mode of bacterial growth in relation to the mechanism of action of the antibiotic (for example, β -lactams require target cells to be actively dividing), (iii) the modes of resistance that are exhibited by colonizing species (for example, enzymatic deactivation through the production of β -lactamases results in a reduced antibiotic concentration generally, whereas the removal of antibiotics from bacterial cells through the action of efflux pumps does not), (iv) the likely airway antibiotic concentration that will be achieved (currently poorly characterized), (v) the impact of concurrent host immune response to infecting bacteria, (vi) drug metabolism and the rate of removal in different patients, and (vii) the impact on antibiotic-resistant species of the removal of antibiotic-sensitive species with which an antagonistic or symbiotic relationship exists.

Further, once antibiotics have been administered, a number of factors compound the difficulty of determining what their impact has been. First, the characteristics of CFPE vary considerably both between episodes within an individual patient and between individual patients. This variability is best illustrated by the fact that no standardized definition for an exacerbation has been universally accepted [34]. In turn, practitioners' definition of the occurrence of CFPE, and the type and timing of treatment initiation, can vary considerably. Second, the multi-factorial nature of CF lung disease, combined with the multimodal treatment of different aspects of the condition, means that clinical signs and patient-reported outcome (PRO) measures (Box 1) can be influenced by a wide range of factors whose relative roles are not easily distinguished. In addition to their antimicrobial roles, some antibiotic treatments also have immunomodulatory activities (e.g. macrolide antibiotics [47]). Others cause side-effects that are difficult to distinguish from the symptoms of CFPE [47-49], implying that antibiotic treatments have an impact on both bacteria and the host. To add yet another layer of complexity, some antibiotics can influence the expression of virulence traits by target microorganisms [1,2,7]. An example of this phenomenon is in the action of aminoglycosides, particularly tobramycin, gentamicin and amikacin [35]. Depending on their concentration, these compounds can be bacteriostatic or bactericidal [50]. At sub-inhibitory levels, aminoglycosides have been shown to promote biofilm development by *P. aeruginosa* and *Escherichia coli* [2], an effect that, in turn, is associated with greater resistance to antibiotic therapy [45].

Box 1. Current assessments of antibiotic treatment efficacy in CF lung disease

Clinical assessments

These include a number of key outcome measures:

- Spirometry: a number of the measures of lung function can be taken. Of these the most widely used is FEV1 (forced expiratory volume in one second) [40,70], not only to help identify exacerbations and determine long-term progression of lung disease [30,71], but also as the primary outcome measure of clinical efficacy [71]. However, measures of lung function, such as FEV1 and FEV6 (forced expiratory volume in six seconds) might not reflect short-term clinical changes [72,73].
- Sputum production and appearance: an increase or new onset of sputum production or a change in the appearance of sputum are associated with CFPE [34], and as such can be used as an outcome measure.

- Temperature: fever can be a symptom of CFPE, and the reduction of elevated body temperature has been used as an outcome measure of treatment [73].
- Respiratory rate: increased respiratory rate is one of the clinical signs associated with CFPE [32], and has been shown to be a sensitive predictor of respiratory dysfunction, being significantly correlated with airway obstruction, hyperinflation, arterial oxygenation, rib-cage–abdomen discoordination, and maximum ventilation during exercise [74].

It is important to note that clinical outcome measures such as these can be affected either by other, non-antimicrobial treatments and the direct effect on the patient of the antibiotics being administered [48]. There is therefore an acknowledged need to include alternative outcome measures that might be more sensitive to disease severity and change following treatment [40].

Patient-reported outcomes (PROs)

PROs are increasingly used to assess treatment outcome in CFPE, and have been validated in CF clinical studies [75]. PROs can be any measure of a patient's health status, elicited directly from the patient, that assesses how the patient 'feels or functions with respect to his or her health condition' [76].

PRO measures in CFPE include assessments of general well-being, as well as specific changes in the amount of sputum produced, breathlessness, and the severity of coughing [75]. These measures can be assessed either by direct communication with the patient, questionnaires, or through recording systems such as visual analog scores (VAS), a system that is widely used and well-validated [77].

Although it is not known whether such effects are commonly encountered with different antibiotics or with clinically important bacterial species, there is clearly a potential that antibiotic gradients within the airways could reduce the viability of some bacterial populations while enhancing antibiotic resistance in others. This means that, as well as affecting the size of the viable or metabolically-active bacterial population, these treatments can affect the way in which these bacteria are perceived by the host, and that a change in clinical signs might result from the impact of antibiotic therapy on the gene expression, as opposed to the viability, of target bacteria.

All these factors greatly complicate the process of predicting the likely impact of any antimicrobial therapy, or of characterizing what the effect of such treatment has been, particularly in chronic and/or polymicrobial infections. However, if the impact of antibiotic treatment on the bacterial populations that chronically infect the airways is measured directly, instead of indirectly via symptoms, then many of these confounding factors could be excluded from the analysis (Figure 2). Further, it is likely that the impact and efficacy of antibiotic treatments in this context will only be determined by the serial collection of detailed and comprehensive data regarding the nature of these infections. Performing these measurements before, during, and after treatment would identify any dynamic trends that might occur. However, in order to inform treatment, the generation of such data should be focused on addressing a number of key questions (Box 2).

Box 2. Assessing antibiotic efficacy in the treatment of CFPE

Key questions that must be addressed by culture-independent strategies in order to inform ongoing treatment:

- Is there a change in the number of viable cells of the pathogen or pathogens being targeted?

- Is there a change in the total number of viable bacteria present in the airways?
- Is there a change in the relative abundance of viable bacterial cells between the different species present?
- Is there a change in the expression of virulence factors or modes of growth associated with CFPE or infection severity?
- How do observed changes in microbial abundance or behaviors relate to changes in symptoms or lung function?

The weighting placed on these questions will vary depending on the nature of the treatment being administered. Data derived from addressing these questions need to be combined with clinical signs and symptoms to provide a complete overview of treatment efficacy.

CFPE as a model of polymicrobial infections

Whilst *P. aeruginosa* is arguably the most clinically-important CF respiratory pathogen, it is not alone in the lungs of CF patients. Culture-based microbiology has identified a number of other species of clinical importance, including *Staphylococcus aureus*, *Burkholderia cepacia* complex, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and several other rarely isolated opportunist Gram-negative species.

Determining the relative abundance of bacterial cells of each of these species in a respiratory sample through culture-dependent approaches is hindered by the presence of bacteria in a viable but poorly-cultivable state. Real-time PCR (RT-PCR) provides a culture-independent approach to quantify changes in bacterial load [51], based on the accurate enumeration of copies of particular genes in a sample. However, whilst RT-PCR can accurately determine the number of gene copies present in the sample, it does not indicate the number of viable cells of a given species. For example, were a treatment to result in all cells of a particular species being killed, the number of gene copies present could remain unchanged in the absence of efficient clearance of material from the site of the infection; thus, the accumulation of dead bacterial cells and extracellular DNA at the site of infection can result in an overestimation of the number of live bacteria that are present. One way to achieve accurate cell enumeration is through the use of propidium monoazide (PMA)-based photo-cross-linking prior to DNA extraction [52]. PMA is a membrane-impermeant dye that selectively penetrates the compromised membranes of dead cells and intercalates into DNA. After intercalation and upon exposure to light, PMA covalently cross-links DNA, and this in turn renders the DNA unavailable for PCR amplification. RT-PCR-based enumeration incorporating this strategy has been shown to reveal decreases in *P. aeruginosa* load in respiratory samples that are not detectable using standard DNA-based RT-PCR (Figure 3) [51].

Clearly, RT-PCR-based assays could be developed to determine numbers of bacterial cells of each of the species commonly isolated from respiratory samples by culture methods, and potentially could provide rapid and accurate data on species-specific viable cell load in the airways during infection dynamics and therapy. However, such a species-specific strategy makes the assumption that these species are of the greatest clinical significance. Although infection with any of these species is potentially clinically important, the above list has been generated through traditional culture-based diagnostic microbiology. An increasing number of studies report a diverse array of bacterial species present in CF respiratory secretions [15,16,53-55], and it is important that the list of species thought to be pathogenic is revisited to identify those species that are directly clinically significant – a key step in selecting

targeted antimicrobial therapy. For example, the significance of strictly anaerobic bacterial species that are increasingly detected in CF respiratory samples [26,53] requires investigation. In addition, defining the composition of the entire bacterial community might also be clinically informative. Diversity leads to the potential for social interactions between microbes [56,57], and these could in turn play a significant role in the way in which individual species behave, in how the integrated microbial community behaves, and in the relationship between host and bacterial community. Studies of polymicrobial systems in other contexts have shown that interactions between species within such bacterial communities can dampen or exaggerate outcomes in relation to pathology and response to therapy [23,58,59]. A study based on *in vitro* model systems of the CF lung have shown the virulence of known CF pathogens, such as *P. aeruginosa*, was increased significantly by the presence of species previously dismissed as clinically insignificant, such as those commonly derived from the oral cavity [58]. Obtaining a comprehensive overview of the bacterial community structure and membership is essential if the impact of therapy on polymicrobial infections is to be understood fully.

As discussed above, attempts to define the wider group of bacterial species associated with the CF lung infections using a range of bacterial community profiling strategies have been made. These studies have revealed a diverse bacterial community [15-20,60,61] with comparable or relatively high abundances of many species in addition to those traditionally recognized as key CF pathogens [16,53,54]. However, it is the application of these analytical tools to samples collected over extended, or clinically significant, periods of time that will reveal most about the processes that drive lung infection [16]. This consideration must be included in the design of future investigations.

Characterizing bacterial behavior *in vivo*

As discussed above, the expression of virulence genes by bacteria colonizing the CF airway can be influenced by a wide range of external factors including the use of antibiotics. This differential expression of virulence presents the possibility of therapy aimed at its amelioration, rather than focused on a reduction of viable population size. Further, changes in gene expression could be predictive of changes in respiratory health and/or response to therapy. A system for relating virulence gene expression to disease was set out by Falkow in his molecular Koch's postulates [62]. However, this requires the levels of expression of these traits at the site of infection to be characterized directly.

There are a number of ways in which such measurements could be achieved. RT-PCR can be used to quantify the expression of genes encoding known virulence factors or virulence-related behaviors. This is done through comparison of levels of reverse-transcribed complementary DNA (cDNA) for the virulence factor to those of house-keeping genes [63]. Where the genes coding for clinically-significant behavioral traits are not known, the total gene expression by a particular bacterial species can be determined through the use of DNA microarray-based analysis [64], from which an expression profile for a variety of virulence-associated genes could be determined concurrently.

Although both gene-specific RT-PCR [65,66] and microarray-based analysis [67,68] have been shown to be useful in characterizing bacterial behavior in a number of different types of infection, only rare successful application of these techniques has so far been achieved in the analysis of CF respiratory infections *in vivo*. This is in part due to a number of important technical considerations, not least of obtaining sufficient quantities of high-integrity mRNA [63]. However, overcoming issues surrounding the validation of appropriate sampling and analytical protocols could allow these highly informative strategies to be applied to the analysis of CFPE.

Predicting clinically-important changes in chronic infections

Antibiotic treatment is initiated once a period of CFPE has been identified. Currently, this judgement is based, to a large extent, on clinical signs. The factors that trigger the onset of CFPE are not as yet known and might include both changes in the infective microbial community and in the host response [21]. However, if CFPE does occur in response to microbial factors, there would probably be a delay before these changes are reflected in clinical signs. If these changes can be identified directly, this could present an opportunity to intervene to prevent the onset of CFPE.

The microbial changes that precede or coincide with the appearance of clinical signs might be as simple as an increase in bacterial load or a change in mode of growth of one or a few key species [69], or as complex as subtle shifts in the gene expression patterns of the community as a whole [4]. Importantly, in each case the techniques described here for the determination of treatment efficacy could be deployed for surveillance of markers that herald changes in infection dynamics. However, only through the collection and analysis of such data in pilot studies will it be possible to determine which of these factors is most useful in the prediction of periods of CFPE.

The application of molecular, culture-independent strategies to the analysis of CFPE has the potential to increase greatly our understanding of this important clinical issue and to influence the treatment of chronic infection. There are, however, a number of other important factors that will require further consideration. These include which clinical outcomes (such as laboratory data, patient- or physician-generated symptoms scores, radiographic scores, lung function measurements, and general quality of life scores, among others) to compare with microbiological data, and how to rank those clinical measures, something that might only be possible with a greater understanding of the mechanism driving CFPE. The extent to which non-bacterial microbes, including fungi and viruses, interact both with the host and the bacterial community to influence infection remains largely undefined. These obstacles will eventually be surmounted, however, and the data that are obtained through the application of molecular analysis to the study of CFPE, and to infections more widely, will provide insight into the mechanisms that drive polymicrobial infections.

Conclusions

Here we describe ways in which molecular diagnostic strategies can be used to more effectively define the identities and behavior of bacterial species in both CF respiratory infections and in polymicrobial and chronic bacterial infections more widely. Clearly, the complexity of data derived through the use of these culture-independent approaches will only increase as technologies such as sequencing advance. The use of such next-generation community profiling strategies provides an opportunity to gain unprecedented insights into the fundamental processes that define such infections. However, factors such as the underlying nature of polymicrobial infections, and the complex relationships between microbial communities and the host as discussed here, must be considered if these novel approaches are to result in improved therapy.

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Figure 1. Diagnostic strategies for acute, single-agent infections as compared to chronic acute infections. Abbreviation: Q-PCR, quantitative-PCR.

Figure 2. Current pathway for determination of CFPE treatment efficacy as compared to direct characterization based on molecular genetic analysis.

Figure 3.

P. aeruginosa loads in sputum sample before and after IV antibiotics, with and without PMA treatment. Sputum aliquots collected before and after 14-day courses of IV antibiotics were either subjected to direct DNA extraction or were first treated with PMA to exclude non-viable cells. Determination of *P. aeruginosa* load was then performed by *P. aeruginosa*-specific RT-PCR. A significant decrease in load was only identified where PMA treatment was employed ($P < 0.05$, paired *t*-test). Error bars represent SEM. Adapted from Ref. [51]. Abbreviation: cfu, colony-forming units.