Antibiotic Resistance of Pathogens Causing Community-Acquired Pneumonia

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SUMMARY

Community-acquired pneumonia remains an important cause of disease and death both in the developed and the developing worlds and therefore continues to have major medical impact. The mortality remains high despite the ready availability of potent antimicrobial

agents to which the organisms are susceptible. However, management of these infections is potentially complicated by the emerging resistance of many of the common pathogens to the different classes of antibiotics that are usually prescribed. Furthermore, it is also being recognized that antibiotic resistance and/or treatment failures may occur not only through traditional microbial antibiotic resistance mechanisms, but also through less well-defined mechanisms, particularly those developed by the microbes in relation to their quorum sensing/biofilm machinery. Much recent research in this field has been focused on evaluating the clinical impact of antibiotic resistance on optimal antibiotic treatment and antimicrobial choices, as well as alternative strategies to deal with antibiotic resistance and/or treatment failures.

Key Words: antibiotic resistance, beta-lactam antibiotics, biofilm formation, community-acquired pneumonia, fluoroquinolones, macrolides, quorum sensing

Introduction

Community-acquired pneumonia (CAP) continues to be associated with significant morbidity and mortality in all regions of the world, with a high clinical and economic burden.[1-7] Short term mortality may be as high as 14% overall, while long term mortality reaches greater than 50% within 5 years.[7] It is the only infectious disease consistently among the top ten causes of death worldwide and in many areas of the globe it is as high as the second most common cause. It is also the second leading cause for hospital admission, after childbirth.[5] Treatment of these infections is potentially compromised by the rapid emergence of resistance of many of the usual pathogens to the most commonly prescribed antibiotics, including beta-lactams, macrolides/azalides and fluoroquinolones.[5]

Even multidrug resistant strains (commonly defined as resistance to 3 or more of the commonly used classes of agents) are begining to emerge. The current manuscript reviews important resistance issues among the most common CAP pathogens, highlighting both the conventional mechanisms of antibiotic resistance as well as resistance development associated with quorum sensing/biofilm formation, cigarette smoking and immunosuppression.

Common pathogens associated with CAP

The common pathogens causing CAP are very similar in many parts of the world, including the United States, Europe, Latin-America, Asia Pacific and sub-Saharan Africa, with a few exceptions.[1-4,8-11] In most areas, the commonest isolate is *Streptococccus pneumoniae* (pneumococcus) and this holds true in cases treated in the outpatient, inpatient, or even the intensive care unit setting.[12,13] It is therefore recommended that this pathogen should always be covered for with empiric antibiotic therapy. Other common pathogens are the so-called atypical pathogens, including *Legionella pneumophila, Chlamydia pneumoniae* and *Mycoplasma pneumoniae,* as well as the respiratory viruses. While these pathogens may cause CAP on their own they are often associated with other common bacterial pathogens, such as the pneumococcus, and this combination of microorganisms is more likely to be associated with a more severe pneumonia. Other pathogens that are commonly found include *Staphylococcus aureus* and some of the gram negative rods. The latter include *Haemophilus influenzae* and *Moraxella catarrhalis,* and in some areas, Enterobacteriaciae and occasionally others. It is clearly beyond the scope of this manuscript to describe completely all the issues in resistance with regard to all

these pathogens, however, some of the more pertinent issues with regard to many of the more common pathogens are highlighted.

Impact of antibiotic resistance on outcome

While much is known about the prevalence, risk factors and mechanisms of antibiotic resistance, an important consideration is whether the presence of antimicrobial resistance alone is associated with adverse outcomes (e.g. death, treatment failure, persistence of infection).[14-16] Less well studied, but certainly not of less concern, has been whether the presence of resistance has any clinical relevance in the treatment of common respiratory infections, such as the pneumococcus, treated with the common classes of antibiotics.[17-19] In some infections, such as meningitis and otitis media, poor outcomes are much more likely to occur in association with antibiotic resistance; however, the evidence for this in CAP is considerably less clear.[14,15,17,18] There are certainly multicentre studies from a number of regions of the world indicating that antimicrobial resistance has little impact on outcome.[1,4] Nevertheless, it is clearly evident that antibiotic resistance is highly prevalent and increasing,[18,20] and that antibiotic prescribing habits have changed as a consequence of perceived problems with resistance.

Antimicrobial resistance among the common respiratory pathogens

There has been considerable worldwide investigation into the prevalence, evolution, mechanisms and risk factors for antimicrobial resistance among the common respiratory pathogens, and particularly the pneumococcus, over a considerable period of time.[14,18,20-24] With this has come the recognition not only that resistance pattens change over time,

but also that there are significant geographical and regional differences in the patterns of resistance such that knowledge of local resistance patterns is essential in guiding the choice of initial empiric antibiotic therapy.[12,18,22-24] Furthermore, more recent understanding of the pharmacokinetic and pharmacodynamic characteristics of the various antibiotics and their relationship to likely outcomes has helped inform decisions regarding antimicrobial therapy and its optimal dosing, particularly in the setting of antibiotic resistance.[17,19,25-27]

Streptococcus pneumoniae

This microrganism has developed resistance to a myriad of antibiotics including the penicillins and cephalosporins (beta-lactams), macrolides and fluoroquinolones, and even multidrug resistance has begun to emerge.[17,18,28-30] Little evidence existed for increased morbidity and/or mortality worldwide in patients with CAP infected with penicillin resistant pneumococci (based on existing breakpoint definitions of resistance) who were treated with penicillins and this lead to the Clinical and Laboratory Standards Institute (CLSI) revising the breakpoints for resistance in non-meningeal isolates in 2008 (susceptible defined as MIC levels $\leq$ 2 μg/ml, intermediate resistance defined as MIC of 4 μg/ml and resistant as MIC of 8 μg/ml).[11,14,29,30] This has resulted in the prevalence of penicillin resistance reported with the pneumococcus to be very much lower.[31] The mechnism(s) of beta-lactam resistance in the pneumococcus are primarily related to alterations in the penicillin binding proteins.[18] The latter are antibiotic target sites, which are integral to cell wall synthesis.[18] It has been said that provided appropriate beta-lactam agents are used in adequate doses, current prevalences and levels of beta-lactam

resistance among peumococci worldwide are such that resistance is unlikely to have any significant impact.[17,18,26,28,29] Numerous studies attest to the ongoing success of beta-lactam therapy.[26,32]

In contrast to that with penicillin, macrolide resistance is a serious and highly prevalent problem worldwide.[30] Two main mechanisms of macrolide resistance exist, namely, low level resistance (MIC 1-32 μg/ml), due to an active efflux pump mechanism (*mef*(A) gene), and high level resistance (MIC > 64 μg/ml) due to a ribosomal methylation mechanism affecting the macrolide binding site (*erm* (B) gene).[18,30] Different proportions of these two mechanisms exist in different parts of the world and in some areas, such as South Africa, both mechanisms may co-exist, translating overall into high-level resistance.

Although failures of macrolide therapy (clinical and/or microbiological) have been documented in the presence of macrolide resistance, these have been relatively few, and there is a rather poor correlation between macrolide resistance and treatment outcome.[17,18,26,30,33-37] This has been described as the "in vivo-in vitro paradox", and there have been various suggestions forwarded to try and explain and understand this phenomenon.[17,33,35,36] Nevertheless, it is commonly recommended that macrolides, as monotherapy for CAP, should be only used in areas where macrolide resistance is known to be low and in patients where risk factors for macrolide resistance are not present.[17] Macrolides, as part of combination antibiotic therapy in more seriously ill patients, and to

cover for so-called “atypical pathogens” in CAP, continue to play an important role in the management of patients with CAP.[11,19,28]

Resistance of the pneumococcus to fluoroquinolones is currently very uncommon, but is increasing worldwide.[18,30,38] Fluoroquinolones inhibit bacterial DNA synthesis by interacting with the intracellular drug target, DNA gyrase and the enzymes topoisomerase IV. [18] Resistance develops by mutations occurring in the quinolone resistance determining region (QRDR), involving *gyr*A and *par*C. In the case of low level resistance, mutations most commonly occur in *par*C, and these isolates test susceptible to the newer fluoroquinolones, whereas in high level resistance dual mutations occur affecting both *par*C and *gyr*A. [18] Efflux pumps are also sometimes found, but the significance of this potential mechanism of resistance is unclear.[18,39] In the presence of high level resistance with dual mutations, treatment failures with macrolides have been reported.[18,37,40] However, what is less well known is that the presence of a first-step mutation in the QRDR, not translating into in vitro resistance, may evolve during treatment and result in the emergence of fluoroquinolone resistant strains during fluoroquinolone therapy.[40] Unfortunately, the prevalence of these strains carrying single-step mutations is unknown in many parts of the world.

Resistance to other antibiotic classes of antibiotics, a number of which are not commonly used or recommended for the treatment of CAP, has been described and is reviewed elsewhere.[18]

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Mycoplasma pneumoniae

Mycoplasmal organisms lack a cell wall and are therefore resistant to cell wall synthesis inhibitors, such as beta-lactams, glycopeptides and fosfomycin. However, in addition to tetracyclines and fluoroquinolones, macrolide antibiotics are effective therapeutic agents against the so-called "atypical pathogens". Macrolides are therefore recommended in many of the international CAP guidelines as first-line choice for the treatment of *M. pneumoniae* infections, which may be a cause of as many as 11-15% of cases of CAP.[41] While macrolide resistance among *M. pneumoniae* has been emerging in children, particularly in countries such as Japan, more recently reports have begun to apppear, documenting the occurrence of CAP in adults due to macrolide resistant *M. pneumoniae.* In one of the first such case reports in adults, also from Japan a 28 year old lady was found to be infected with an isolate of *M. pneumoniae* with elevated MICs for the various macrolide/azalide/telithromycin antimicrobial agents, due to the same point mutation (A to G transition) at position 2063 in domain V of the 23S rRNA gene that accounted for > 90% of cases in the pediatric population in Japan.[42] This mutation is associated with the highest level of resistance to the macrolide group of drugs.[43] Further case studies have been reported and other mutations documented, although these are less common.[44] Interestingly, none of the *M. pneumoniae* strains has been found to be resistant to either minocycline or the fluoroquinolones. Such strains have also subsequently been reported from other countries, including France, USA, Denmark and China.[43,44] More than 90% of isolates in China are resistant to erythromycin and azithromycin.[45] The subject of mycoplasmal antibiotic susceptibility and resistance has been extensively reviewed elsewhere.[45] The exact reason(s) for the emergence of these strains is unclear, but may

relate to the use of low dose macrolides in chronic lung diseases, or possibly other reasons.[44] The clinical course of these infections, especially the duration of fever and duration of cough in these patients, is prolonged and most cases had been treated with macrolides previously.[43] In most situations, therapy in these cases has been changed to minocycline or a fluoroquinolone, with good clinical outcomes, although neither of these agents is ideal for children.

Haemophilus influenzae and Moraxella catarrhalis

H. influenzae is a common colonizer of the pharynx, even in healthy individuals, and may colonize the lower respiratory tract in patients with underlying respiratory disorders.[46] While this microorganism may cause upper respiratory tract infections (URTI) (otitis media and sinusitis), and it plays a significant role in acute exacerbations (AE) of COPD, it may also be a cause of up to 15% of cases of CAP.[46] Since the 1970s antibiotic resistance in this pathogen has been increasing. Similarly, *M. catarrhalis* has only more recently been recognized to be an important human pathogen, and is now recognized to be the second most important cause of AE of COPD, an important cause of URTI (sinusitis and otitis media) and also to cause up to 10% of cases CAP, especially at the extremes of age.[46]

Resistance to both these microorganisms is commonly due to due to emergence of beta-lactamase production. This was initially reported as TEM-1 type in the case of *H. influenzae* and later a novel new beta-lactamase was found that was termed ROB-1.[47] There is also a suggestion that some isolates are also beta-lactamase resistant, but do not

carry either TEM-1 or ROB-1 and therefore appear to have a novel beta-lactamase type.[47] In the case of *M. catarrhalis* the beta-lactamases are most commonly those termed BRO-1, BRO-2 or BRO-3.[46] The prevalence of resistance strains varies across the world.[46,47] For example, in the PROTEKT Study (a surveillance study investigating antimicrobial susceptibility of respiratory pathogens around the world) there was a marked variation in the range of beta-lactamase production in the different regions in the case of *H. influenzae* (from 1.8% in Italy to 65% in South Korea), whereas 92% of all the isolates of *M. catarrhalis* in that study were found to be beta-lactamase positive.[46]

Other types and mechanisms of antibiotic resistance in the case of *H. influenzae* have been documented and extensively reviewed elsewhere.[47] Strains of *H. influenzae* have also been documented to be resistant to ampicillin, but not due to production of beta-lactamases (beta-lactamase negative ampicillin resistance (BLNAR) strains), and the mechanism is thought to be due to alterations in the pencillin binding proteins (PBPs).[47] These strains appear to be more common in Japan and France.[47] Some strains have been found containing both these mechanisms of resistance (so-called BLPACR strains).[47] It is rather worrying that in the PROTEKT study some *H. influenzae* isolates demonstrated intermediate resistance to macrolides, while most of the *M. catarrhalis* strains were sensitive to all the other antibiotics tested.[46] Subsequently, a macrolide efflux pump mechanism was documented in *H. Influenzae*, although there is some debate about the clinical significance of this, since the isolates still have rather low MICs.[47] Tetracycline resistance among these pathogens appears also to be due to an efflux pump and resistance to the fluoroquinolones is still very uncommon.[47] Thus in the case of *H. influenzae* some

caution needs to be exercised with the use of certain beta-lactams, as well as macrolide/azalides and tetracyclines, at least in certain areas.[47]

In the case of *M. catarrhalis,* beta-lactamase appears to be the main mechanism of resistance and so these pathogens remain susceptible to most of the other agents potentially used for CAP other than ampicillin/amoxicillin, including cephalosporins, macrolides/azalide, tetracycline and fluroquinolones.[48,49]

Staphylococcus aureus

S. aureus is an important cause of various severe infections in humans, including pneumonia. Methicillin-resistant isolates of *S. aureus* (MRSA) are uniformly resistant to all available penicillins and other beta-lactam agents. While infections with such microorganisms have classically been prevalent in the setting of the hospital environment, in other health-care facilities and in individuals with contact with such environments, since the 1990s there has been an explosion of patients with MRSA infections who do not have classical risk factors of contact with the hospital system and increasing recognition of the new strains of so-called community-associated MRSA (CA-MRSA) infections.[50-52] These infections have been extensively reviewed elsewhere.[52,53] Such pathogens seem to be more common in the USA than in Europe (11). CA-MRSA isolates are resistant to fewer non-beta-lactam drugs than HA-MRSA strains and in the USA the strains most commonly associated with these infections (USA 300 and USA 400) frequently carry Panton Valentine Leukocidin (*pvl*) genes. Strains producing PVL

have also often been shown to be avid producers of biofilm,[50] which may itself may limit the efficacy of antibiotics (see below).

Molecular means (multilocus sequence typing (MLST), pulsed field gel electrophoresis (PFGE) and toxin gene expression) have allowed the differentiation of CA-MRSA from their healthcare-associated (HA)-MRSA counterparts although some MRSA infections with onset in the community have been of the HA-MRSA type.[52,53] Cases with MRSA are typed for SCC*mec*, which is the mobile genetic element that carries the *mec*A gene which codes for methicillin resistance.[52] SCC*mec* is classified into types (based on *mec* and *ccr* types) and each type is further classified into subtypes based on differences in the so-called junkyrd (J) region.[52] HA-MRSA usually carry larger SCC*mec* types (I, II and III), whereas CA-MRSA are usually smaller (types IV, V, or VII).[52] The former carry additional beta-lactam resistance genes allowing them to survive more easily in the hospital environment.[52] PVL genes are more common in CA-MRSA. PVL has a myriad of toxic and pro-inflammatory activities and it appears that these may possibly play a major role in the pathogenesis of CA-MRSA infections, although there is some debate about this issue and other virulence factors may also be important in this respect.[52]

These infections are now recognized as an emerging clinical entity with distinctive clinical features and high morbidity and mortality.[52] CA-MRSA infections tend to occur more commonly in younger, healthy patients and while they mainly cause skin and soft tissue infections, they may also cause a necrotizing form of pneumonia.[11,51,53,54] CA-MRSA infections have become commonplace in the USA, accounting for > 50% of

staphylococcal infections in the outpatient setting.[51] They have also become more common in other parts of the world, although in some areas of the globe they have remained either rare or almost non-existent.[50,51,53] Classically described is an influenza or influenza-like prodrome, followed by acute shortness of breath, sepsis and hemoptysis.[52] However, some studies have indicated that these infections do not necessarily follow influenza infections but a number of patients appear to have underlying immunocompromizing conditions (HIV infection, diabetes mellitus, leukaemia, steroid therapy, immunoglobulin disorders).[54] No clinical features are absolutely characteristic of these infections, but the demonstration of a necrotizing pneumonia on chest radiograph and a rapidly increasing pleural effusion should raise the suspicion.[54]

These infections are more difficult to treat than infections caused by methcillin-sensitive strains of *S. aureus,* since there are relatively few agents available to treat such infections, with some of these agents having significant limitations, and strains have emerged that are resistant to each of the agents that are theoretically available to treat such infections.[53] Vancomycin or linezolid have been recommended for initial therapy.[52] However, failures of vancomycin have been documented even with isolates which according to MIC testing were susceptible.[52] Most studies have documented a high mortality rate and improved outcomes appear to be associated with the use of antimicrobials that inhibit exotoxin production and/or non-toxigenic strains, i.e. clindamycin or linezolid.[54] It is therefore most commonly recommended that in the case of those strains producing PVL, combination antibiotic therapy is used, with an agent or agents that are able to decrease toxin production, such as linezolid and clindamycin.[51,52,54]

Gram-negative rods

In some areas of the world, gram-negative enteric bacteria, such as *Klebsiella pneumoniae, Escherichia coli* and various other Enterobacter species appear to be relatively common causes of CAP.[4,55] Over a number of years there has been a progressive increase in antimicrobial resistance among many of these gram-negative pathogens, including the presence of extended spectrum beta-lactamase (ESBL) production, and although this has been more common among nosocomial infections, cases have also been noted in patients with healthcare-associated pneumonia, as well as those with community-acquired infections, particularly in certain areas of the world.[56-59] Such infections are known to be associated with an increase in mortality, particularly among patients with pneumonia, and specific therapy, such as with ertapenem, is required for effective treatment.[60] Various other forms of antimicrobial resistance have been documented in gram-negative pathogens overall.[56]

Alternative mechanisms of microbial resistance

The following sections will address the roles of microbial biofilm in particular, as well as exposure of bacteria to environmental mutagens or immunosuppression in promoting treatment failure and/or resistance to antibiotics. This is followed by a consideration of non-antibiotic strategies to overcome resistance.

Biofilm

Encasement in biofilm is a survival strategy used by bacterial pathogens including staphylococcus and streptococcal species, *Mycobacterium tuberculosis, E. coli, K.*

pneumoniae, and *P. aeruginosa,* as well as yeasts and fungi such as *Candida albicans* and *Cryptococcus neoformans*.[61-64] Biofilm, which has been implicated in 60-80% of all microbial infections,[65] is a hydrated, self-generated, well-ordered matrix of extracellular polymeric substances, mainly polysaccharides, proteins and DNA, with the exception of mycobacterial species, which utilize mycolic acids as a major constitutent of biofilm.[63,66] When embedded in biofilm, either attached to epithelial surfaces or sequestered intracellularly, microbial pathogens are effectively insulated not only against antibiotics, but also against the cellular and humoral defense mechanisms of the host. In this relatively quiescent state, the bacteria can re-emerge and re-populate previously colonized sites on removal of stressors, or at times when host defenses are transiently compromised as may occur during infection with influenza virus, respiratory syncytial virus or HIV-1.[61,62,67]

Although biofilms may contain a single bacterial strain, most naturally occurring biofilms contain multiple bacterial species and are known as inter-species, multi-species, or polymicrobial biofilms.[62,68,69] Co-existence in polymicrobial biofilms is not necessarily harmonious, however, due to competition for nutrients, as well as for optimally aerated and irrigated sites. On the other hand, cooperative interactions, especially those which promote resistance to antibiotics, reinforce the overall resilience of the biofilm community.

Quorum sensing and biofilm formation

Although the exact molecular mechanisms underpinning biofilm formation remain to be conclusively established, quorum sensing mechanisms activated in response to environmental stress, including antibiotics, and phagocyte-derived reactive oxygen species (ROS) such as hydrogen peroxide, appear to mediate the transition from planktonic to biofilm growth,[62,65,70,71] with approximately 4-10% of the bacterial genome and ≥20% of the proteome being regulated by quorum sensing mechanisms.[65]

Quorum sensing is a process by which microorganisms communicate using chemical messengers known as autoinducers (AIs), usually small soluble molecules or peptides.[65,72] This signalling process enables bacterial communities to coordinate their gene expression according to cell density, promoting the transition to diverse phenotypes on reaching a critical threshold concentration.[62] Depending on the local microenvironment, quorum sensing systems may upregulate processes which augment virulence, enabling an orchestrated onslaught on the host. Alternatively, as mentioned above in a hostile microenvironment, quorum sensing systems can promote the transition to a less aggressive, persistent state via biofilm formation.

Gram-negative bacteria most commonly utilize type 1 family autoinducers (AI-1) known as N-acylated-L-homoserine lactones (AHLs), of which 3-oxo-hexanoyl-homoserine is the prototype,[72] as their primary inducers of quorum sensing.[65,72] These are small, soluble, diffusible molecules which vary with respect to their acyl substituents.[74] They are produced by AHL synthases belonging to the family of LuxI-type proteins.These

utilize *S*-adenosylmethionine and an acyl-acyl carrier protein to synthesize AHLs, which in turn interact with their cytoplasmic receptors, the LuxR-type proteins, AHL-regulated transcription factors.[65,72,75-77] Meaningful LuxR-type receptor binding occurs on attainment of a high intracellular concentration of AHLs. The AHL/LuxR-type protein complex usually dimerizes and interacts with the LuxR box in the promoter region of target genes, initiating transcription of quorum sensing controlled genes.[65,72,74] Over 100 types of gram-negative bacteria utilize Lux1/LuxR-type systems to mediate quorum sensing, with many species having multiple Lux1/LuxR-type pairs which target different and common genes controlled by quorum sensing mechanisms, including those involved in biofilm formation in pathogens such as *P. aeruginosa* and *Burkholderia cepacia complex*.[65,74,78]

Gram-positive bacteria, on the other hand, do not produce AHLs, but typically utilize small post-transtationally modified cyclic oligopeptides to initiate quorum sensing. These, in turn, interact with the sensor element of a histidine kinase two-component signal transduction system.[65] Examples of these are the competence-stimulating peptides and cyclic thiolactone oligopeptides of streptococcal and staphylococcal species respectively, both of which are involved in biofilm formation.[65,78,79]

Autoinducer-2 (AI-2) is a recently described family of quorum sensing molecules utilized by both gram-negative and gram-positive bacteria, apparently to mediate interspecies communication, in contrast to AHLs and cyclic oligopeptides which mediate intraspecies communication.[75,80,81] The LuxS protein, the product of the widely conserved *luxS* gene

found in over 70 species of bacteria, mediates the synthesis of 4,5-dihidroxy-2,3-pentadione, the precursor of a pool of AI-2 molecules.[75] The AI-2-mediated sensing mechanism is well-described in *Vibrio harveyi*, and is likely to be broadly operative in other types of bacteria. It requires two proteins, LuxP and LuxQ, which are the periplasmic binding and sensor kinase proteins respectively.[75] These two proteins are thought to form a dimer (LuxP/Q), which is modified following the binding of AI-2 at critical threshold levels to generate LuxR, described by Galloway *et al.* as "the quorum sensing master regulator that controls expression of the genes in the quorum sensing regulon".[75,82]

The involvement of the AI-2 quorum sensing system in biofilm formation has been described in various gram-positive and gram-negative bacterial pathogens including *S. mutans, S. pneumoniae, S. aureus, S. epidermidis, Enterococcus faecalis, E. coli, H. influenzae, Helicobacter pylori, Campylobacter* and *Salmonella* species.[83-86] Because it promotes bacterial persistence and antibiotic resistance via interspecies communication, the AI-2 quorum sensing system is considered to be a potentially exciting target for treatment of polymicrobial infections.[85]

Biofilm and antibiotic resistance

Bacterial biofilms provide an environment which is eminently conducive to minimizing or even negating the efficacy of antibiotics, being achieved by both genetic and passive mechanisms. The genetic basis of biofilm-mediated antibiotic resistance involves inter-

dependent innate and induced resistance mechanisms,[87] while several mechanisms of passive resistance have been described.

Biofilm-mediated innate resistance

Notwithstanding the processes involved in formation of biofilm, innate resistance is primarily a function of the mechano-physical properties of biofilm which impede the diffusion of antibiotics, nutrients and oxygen. In the case of antibiotics, the MIC and MBC values of these agents for biofilm-embedded bacteria may be up to 100-1000-fold higher than those of planktonic bacteria,[63,88] with exposure to low, tolerable concentrations of antibiotics clearly favouring development of genetic drug resistance mechanisms. In the case of nutrients and oxygen, the creation of poorly-aerated, nutrient-depleted zones in the core of the biofilm community results in a slow-down in metabolic activity and growth, resulting in phenotypic resistance.[62]

Biofilm-mediated genetic resistance

As mentioned above, by retarding the penetration of antibiotics, biofilm-associated innate antibiotic resistance may facilitate the induction of expression of resistance genes. In addition, the close proximity of bacterial cells within the biofilm provides an environment amenable to horizontal gene transfer, which can lead to acquisition of antibiotic resistance.[89] Indeed inter-strain and inter-species horizontal gene transfer have been demonstrated in biofilms,[90,91] including transfer of a multi-drug resistance plasmid in *E. coli* biofilms.[77] Although ominous, the clinical significance of biofilm-associated

intra-/inter-strain and inter-species,[92] horizontal transfer of antibiotic resistance genes remains to be established.

Biofilm-associated passive acquisition of antibiotic resistance

Passive resistance to β-lactam antibiotics, unrelated to impaired penetration, has been described for *S. pneumoniae* in polymicrobial biofilms. Budhani *et al.*, using a continuous culture biofilm system *in vitro*, reported that a β-lactam-sensitive strain of the pneumococcus was protected by co-culture with a β-lactamase-producing strain of *M. catarrhalis*.[93] Similarly, Weimer *et al.*, using a chinchilla model of biofilm-associated experimental otitis media, demonstrated that a β-lactamase-producing strain of non-typeable *H. influenzae* also conferred passive protection against amoxicillin on a β-lactam-sensitive strain of the pneumococcus.[94]

Environmental mutagens and antibiotic resistance

Although mutagenic in the Ames test, which utilizes auxotrophic strains of *Salmonella typhimurium*, the potential of environmental mutagens to cause antibiotic resistance by promoting point mutations in bacterial genes encoding proteins which are targets for antibiotics is largely unknown. Very recently, however, Miyahara and colleagues, investigated the effects of exposure of a rifampicin- and ciprofloxacin-sensitive reference strain of *P. aeruginosa* to a series of known mutagens on the development of antibiotic resistance.[95] The mutagens selected for investigation were: i) the known alkylating agents, ethyl methanesulfonate (EMS) and N-nitroso-*N*-methylurea (MNU); ii) benzopyrene (BP) and 1,6-dinitropyrene (1,6-DNP), both products of combustion; iii) N-

nitroso-nornicotine (NNN), a constituent of cigarette smoke; and iv) 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU), an anti-cancer agent. All were used at mechanistically (induction of point mutations) or environmentally-relevant concentrations.[95] The bacteria were exposed to the test mutagens for 24 hours, followed by plating-out and selection of rifampicin- and ciprofloxacin-resistant mutants from which DNA was extracted, amplified by PCR, sequenced and analysed for point mutations in target genes, these being: i) *2-poB*, the β-subunit of RNA polymerase; ii) *gyr A* and *gyr B* which encode the A and B proteins of DNA gyrase; iii) *par C* and *par E* which encode the A and B subunits of topoisomerase IV; and iv) *nfxB* and *mexR*, fluoroquinolone efflux pump regulatory genes.

Exposure of *P. aeruginosa* to EMS, MNU, or BCNU in particular, significantly increased the incidence of resistance to rifampicin, with lesser effects observed with 1,6-DNP, while BP and NNN were relatively ineffective.[95] Acquisition of resistance was associated with point mutations in about 93% of the rifampicin-resistant isolates.[95] Essentially similar effects of these mutagens, albeit at an approximately 10-fold lower frequency, on development of ciprofloxacin resistance were observed, with mutations detected mostly in the *gyrA/B* and *parE* genes, but not *parC, nfxB* or *mexR*.[95]

Although the cigarette smoke-derived mutagen, NNN, was relatively ineffective in promoting antibiotic resistance in *P. aeruginosa*,[95] this may have resulted from the relatively short exposure of the pathogen to the mutagen, as opposed to the prolonged

exposure to multiple mutagens in the setting of active, and even passive, cigarette smoking.

In addition, cigarette smoke, and possibly other environmental toxins, have been reported to induce biofilm formation by bacterial pathogens. Exposure of the oral pathogen *Porphyromonas gingivalis* to cigarette smoke extract *in vitro,* was found to initiate biofilm formation,[96] while inhalation of cigarette smoke by humans is associated with the induction of sinonasal microbial biofilms.[97] In the latter study, Goldstein-Daruech *et al.* isolated various species of bacteria, including amongst others, *S. aureus, S. pneumoniae, K. pneumoniae* and *P. aeruginosa,* from the sinonasal cavities of smokers and non-smokers. Exposure of the majority (12/16) of bacterial isolates from the sinonasal cavities of smokers to smoke generated by the combustion of 5 reference cigarettes *in vitro,* followed by incubation for 17 hours, resulted in vigorous biofilm formation.[97] Similar effects using bacteria isolated from non-smokers necessitated repetitive smoke exposure over a 4 day period. The findings of these two studies[96,97] are compatible with a role for cigarette smoke-induced biofilm formation in promoting bacterial persistence in the setting of impaired efficacy of antibiotics, which may be compounded by its mutagenic potential.

Immunosuppression and failure of antibiotic therapy

The critical dependence of cooperative interactions between antibiotics and host defenses in the eradication of microbial pathogens is underscored by the limited efficacy of these agents in patients with primary immunodeficiency disorders, as well as those with

acquired immunodeficiency. Notwithstanding iatrogenic immunosuppression and HIV infection, old age is one of the most common and increasing causes of acquired immunodeficiency and possible failure of antibiotic therapy, while cigarette smoking interferes with host defenses in the airways.

In the case of the elderly, immunosenescence compounded by multiple comorbid diseases and possibly undernutrition, may interact to complicate the clinical course of infectious diseases, especially pneumonia, urinary tract infections, severe sepsis, meningitis, infective endocarditis, septic arthritis and tuberculosis, often necessitating hospitalization.[98-101] In the hospital setting, administration of antibiotics, which may be frequent and empiric, predisposes the elderly to the emergence of highly resistant pathogens such as methicillin-resistant *S. aureus*, β-lactam-resistant pneumococci, vancomycin-resistant enterococci, and multiple drug-resistant gram-negative bacilli.[100,101] While not directly promoting antibiotic resistance, age-related immunosuppression may favour its development by contributing to treatment failure.

Cigarette smoking is also a well-documented risk factor for respiratory bacterial infection including tuberculosis[102] and severe pneumococcal disease,[103] which is associated with interference with innate and adaptive pulmonary host defenses. In the case of innate immunity, cigarette smoking has been reported to compromise the protective functions of ciliated respiratory epithelium,[104] and upper airway and tracheobronchial epithelial cells,[105,106] as well as with the phagocytic activity of pulmonary macrophages.[107] In an animal model of experimental infection, exposure to cigarette smoke was found to

interfere with the pulmonary T-cell responses to both *M. tuberculosis* and influenza virus.[108]

Although unproven, interactions involving mutagenic potential, induction of biofilm formation, and interference with pulmonary host defenses may limit the efficacy of antimicrobial therapy in cigarette smokers,[102] and possibly contribute to the development of antibiotic resistance. The mechanisms by which smoking may promote treatment failure and resistance are summarized in Table 2.

Non-antibiotic strategies to overcome resistance

These include implementation of: i) more efficient and extensive immunization programmes targeted at high-risk groups, especially the elderly; ii) improved hygiene, nutrition and social upliftment strategies; and iii) heightened awareness of regional antibiotic resistance frequencies in association with discerning antibiotic prescribing practices.[109] In addition, strategies which target biofilm formation and/or its disruption, as well as those which augment innate immunity have the potential to counteract treatment failure and development of antibiotic resistance.

Biofilm-targeted strategies

Two major strategies have attracted recent attention. These are: i) the design and synthesis of pharmacological agents and monoclonal antibodies which inhibit the synthesis of biofilm-inducing quorum sensors, or which antagonize their receptors on

bacterial cells; and ii) the identification of non-toxic chemicals and enzymes which promote disruption of biofilm.

Inhibitors of quorum sensing

As mentioned above, the quorum sensing systems most widely, but not exclusively, utilized by gram-negative bacteria involve the synthesis of AHL and AI-2 autoinducers,[65,75] both of which converge on LuxR, making this an attractive generic target for development of anti-biofilm strategies. Additional strategies include development of: i) inhibitors of Lux 1-type acyl synthases, as well as LuxS-mediated synthesis of AI-2; ii) antagonists of the interaction of AHL and AI-2 autoinducers with their receptors on Lux R-type and LuxP-type proteins respectively; and iii) strategies which broadly inactivate this family of autoinducers.

Modulation of the AHL and AI-2 quorum sensing pathways by small molecules in gram-negative bacteria has recently been described in considerable detail by Galloway and colleagues.[75] These include analogues of AHLs and *S*-adenosylmethionine, which target type 1 AI, as well as cinnamaldehyde and furanones which target both types of AI and are potentially useful in eradicating polymicrobial biofilms containing gram-negative and gram-positive bacteria.[65,75,85,110,111] Other types of compound have also been reported to inhibit biofilm formation by undefined, possibly antimicrobial, mechanisms. These include aryl 2-aminoimidazoles and their derivatives in the case of gram-negative bacteria,[112,113] and 3-arylideneflavonones which alter the integrity of the outer membrane in gram-positive bacteria, possibly antagonizing the uptake of AIs,[114] and pyrroloindoline triazole amides, which appear to antagonize indole metabolism.[115] Indole functions as an

intracellular signal regulating various phenotypes in both gram-negative and gram-positive bacteria, including biofilm formation.[115] Hamamelitannin, an anti-oxidant derived from witch hazel, has been found to antagonize the effects of the cylic thiolactone autoinducing peptide of *S. aureus* on biofilm formation, but toxicity and lack of selectivity may restrict its therapeutic potential.[116]

Although most of these small molecules have demonstrated efficacy in both *in vitro* and *in vivo* models of experimental biofilm infection, usually in combination with antibiotics,[79] their efficacy in the clinical setting remains unproven. Nonetheless the approach is innovative and promising.[75]

Monoclonal antibodies which target the cyclic oligopeptide AIs of Gram-positive bacteria also have the potential to antagonize biofilm formation, and have indeed demonstrated efficacy in a murine model of experimental infection with *S. aureus*.[117] In the clinical setting, however, the therapeutic application of anti-biofilm monoclonal antibodies may be restricted due to issues such as expense, susceptibility to proteolytic cleavage, poor penetration into biofilms, and selectivity for a single, as opposed to a range, of AIs. On a more positive note, however, crystallographic analysis of monoclonal antibody/cyclic oligopeptide AI interactions may reveal structural interactions which would facilitate the development of broadly cross-reactive antibodies or vaccines, at least in the case of *S. aureus*.[117]

Biofilm-disrupting strategies

These are generally based on the use of enzymes which cleave the major components of biofilm (polysaccharidases, proteinases, nucleases), as well as oxidizing agents and surfactants/detergents.[118,119] Because of the multi-constituent nature of biofilm, which varies considerably between species, these agents are generally used in combination. However, their lack of selectivity, together with their pro-inflammatory and pro-allergic potential, underpins their primary use as disinfectants as opposed to therapeutic agents. A notable exception is the use of nebulized DNAase for prevention of *P. aeruginosa* biofilms in the airways of patients with cystic fibrosis.[59]

Potentiation of innate immunity

Although largely untested in the therapeutic setting in humans, augmentation of innate immunity has been identified as a strategy to increase the efficacy of antimicrobial chemotherapy and possibly counter antibiotic resistance.[120-122] Two such strategies involve the use of inhibitors of cyclooxygenase 1 (COX1) or antagonists of Toll-like receptors (TLRs).

Inhibitors of COX1

COX inhibitors effectively attenuate the production of 3'-5'-cyclic adenosine monophosphate (cAMP) by cells of the innate immune system. They do so by inhibiting the production of prostaglandins (PGs) E_2 and D_2, attenuating their autocrine interaction with adenylyl cyclase–activating EP2/4 and DP1 receptors on phagocytic cells. Cyclic

AMP, which has been described as the "master regulator of innate immune cell function,"[123] is a potent inhibitor of FcγR-mediated phagocytosis, as well as production of antimicrobial ROS, and release of antimicrobial peptides/proteins.[123]

Stables *et al.*, using a murine model of experimental group B Streptococcus-induced peritonitis, reported that intraperitoneal administration of inhibitors of COX1, but not COX2, as well as an antagonist of EP2/4 and DP1 receptors, either 1 hour pre- or post-induction of experimental infection, was associated with decreased bacterial survival in the setting of increased phagocytic activity of isolated peritoneal leukocytes, as well as enhanced production of ROS.[120] Similarly, oral administration of a single 500 mg tablet of naproxen to adult human volunteers, followed one hour later by *ex vivo* determination of killing of a penicillin-sensitive strain of *S. pneumoniae,* using a whole blood assay, revealed increased phagocytic activity, generation of ROS and antimicrobial activity.[120] Similar effects on antimicrobial activity were observed using a penicillin-resistant strain of *S. pneumoniae,* although the effects of combinations of penicillin and COX1 inhibitors were not tested.[120] These authors advocate "the potential of inhibitors of PG signalling pathways as adjunctive therapies, particularly in the context of antibiotic resistance."[120] On a more cautious note, however, the augmentative effects of agents which interfere with cAMP-mediated control of inflammatory responses may predispose to inflammation-mediated tissue damage and organ dysfunction.

Agonists of Toll-like receptors

Agonists of TLR2 and TLR4 in particular may be useful in potentiating innate immunity by increasing the synthesis of pro-inflammatory cytokines, especially interleukin(IL)-1, IL-6, IL-8 and TNF by cells of the innate immune system, as well as by epithelial cells.[121,122] However, the use of this type of immunomodulatory strategy, as well as the direct administration of recombinant cytokines such as interferon-γ and granulocyte colony-stimulating factor, or host-derived antimicrobial peptides is likely to be complicated by an inability to selectively target the microbial pathogen. In this setting, inappropriate inflammatory responses are likely to be potentially damaging as opposed to protective.

Conclusion

This review highlights many of the issues associated with antimicrobial resistance and antibiotic treatment failure. Both of these can only be countered using a combination of strategies. Clearly, foremost are the development of novel antimicrobial agents and more discerning use of existing agents. Others strategies include disease prevention targeting high-risk groups, particularly the elderly, more aggressive and effective anti-smoking campaigns, and development of pharmacological and immunological adjuvants which complement antibiotics, in particular those which target biofilm. It is reassuring that the threat posed by biofilm infections is well-recognized, and that innovation, particularly in the development field of small molecule antagonists of biofilm formation, show considerable promise.

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Table 1: Antibiotic resistance among common respiratory pathogens causing community-acquired pneumonia*

Microorganism	Antibiotic class	Common mechanisms of resistance
Streptococcus pneumoniae	beta – lactam	▪ alterations in penicillin-binding proteins (PBP)
	macrolide	▪ efflux pump (*mef* A gene) ▪ ribosomal merthylation mechanism (*erm* B gene)
	fluoroquinolone	▪ mutations in fluoroquinolone resistance determining regions (*par* C and *gyr* A) ▪ (efflux pump)
Mycoplasma pneumoniae	macrolide	▪ point mutation in domain V of the 23S rRNA gene
Haemophilus influenzae	beta-lactamase	▪ beta-lactamase production (TEM-1, ROB-1, and poorly defined other) ▪ (alteration of PBP) ▪ (macrolide efflux pump)
Moraxella catarrhalis	beta-lactamase	▪ beta-lactamase production (BRO-1, BRO-2, BRO-3)
Staphylococcus aureus	"methicillin"	▪ *mec* A gene
Gram negative rods	various	▪ beta-lactamase production, including extended spectrum beta-lactamase (ESBL) ▪ various other mechanisms

*Common mechanisms of resistance are highlighted and some of the less common mechanisms are indicated in brackets

Table 2: Mechanisms by which cigarette smoking may predispose to antibiotic treatment failure and resistance

Mechanism	Effect
• Mutagenic activity	Mutations in antibiotic target genes[95]
• Induction of biofilm formation	Impaired access of antibiotics to target pathogens[96,97]
• Immunosuppression	Dysfunction of the mucociliary escalator;[104] Impaired cytokine secretion by airway epithelial cells;[105,106] Decreased phagocytic activity of alveolar macrophages;[107] Impaired activation of T-cells by airway pathogens.[108]