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Targeted Treatment for Bacterial Infections: Prospects for Pathogen-Specific Antibiotics Coupled with Rapid Diagnostics

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Abstract

Antibiotics are a cornerstone of modern medicine and have significantly reduced the burden of infectious diseases. However, commonly used broad-spectrum antibiotics can cause major collateral damage to the human microbiome, causing complications ranging from antibiotic-associated colitis to the rapid spread of resistance. Employing narrower spectrum antibiotics targeting specific pathogens may alleviate this predicament as well as provide additional tools to expand an antibiotic repertoire threatened by the inevitability of resistance. Improvements in clinical diagnosis will be required to effectively utilize pathogen-specific antibiotics and new molecular diagnostics are poised to fulfill this need. Here we review recent trends and the future prospects of deploying narrower spectrum antibiotics coupled with rapid diagnostics. Further, we discuss the theoretical advantages and limitations of this emerging approach to controlling bacterial infectious diseases.

Keywords

Antibiotic; narrow-spectrum; molecular diagnostics; resistance; bacteria; pathogens

1. Introduction

The advent of antibiotics in the early twentieth century catalyzed a medical revolution, drastically reducing mortality due to bacterial infections. Along with numerous other advances in healthcare, such as vaccines and improved sanitation, antibiotics have contributed to an extension in the average life expectancy in the USA from 59.7 years in 1930 to 78.7 years in 2010.[1] Antibiotics are a critical component of a number of modern medical procedures, including many surgeries and transplants, as the rate of severe complications and death from infection would otherwise be unacceptably high. Decades of

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research into antibiotic development have produced highly effective and safe antibiotics, giving clinicians a wide range of tools to prevent and fight bacterial infections. However, resistance has inevitably followed the release of each new drug,[2–7] and the rapid propagation of resistant pathogens has become a serious issue, resulting in at least 23,000 deaths in 2013 in the United States alone.[8] Of particular concern are the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) which have high levels of resistance and frequently "escape" eradication by antibiotics.[9,10] While drug resistance or insensitivity can arise in other therapeutic areas, only in infectious diseases is that resistance known to be directly transmissible from person to person. Conservative use of antibiotics to slow resistance can be beneficial to society by extending the lifetime of current drugs[11] but doctors still use them liberally to treat individual patients if alternative options are not available. This has created a unique situation where everyone is at risk of having fewer treatment options than in previous decades unless the development of new antibiotics keeps pace with the development of resistance.

Unfortunately, bringing new antibiotics into the clinic has proven to be challenging for a number of reasons. The most fruitful strategy to discover novel antibiotics has been natural product screening, but it is increasingly rare to find compounds with new scaffolds, with rediscovery of known antibiotics posing a significant challenge.[12,13] Synthetic small molecule libraries have not fared better as effective antibiotics tend to have different properties than drugs in other therapeutic areas. Antibiotics often contain complex structures with multiple stereocenters and can be much larger than the usually small, flat compounds that dominate synthetic libraries.[14] Thus, compound collections designed around Lipinski's "rule of five" are poor sources for lead compounds.[15] This has been especially evident in screening campaigns against essential targets identified through genomics in which hits from *in vitro* screens frequently failed to display activity in whole cell assays.[16] A frequently cited factor for the decline in antibiotic development is the potential for low financial returns.[17–19] Bringing new antibiotics to market is seen as a poor investment as they are often reserved as drugs of last resort with a short duration of treatment, yet are subject to a pricing scheme dictated by a market saturated with the generics of older antibiotics that are often still effective.[20,21] Additionally, FDA regulations have made clinical trials for antibiotics difficult and expensive to perform.[22] Non-inferiority trials are usually required due to the ethical constraints of withholding antibiotics from patients with serious infections, and the very low non-inferiority margin requirements result in the need for large patient populations.[20,22] This is exacerbated by the disqualification of patients who have received any prestudy antibiotic, although the FDA has started to ease the regulatory restrictions for antibiotic clinical trials over the last several years. These factors had two major consequences. First, pharmaceutical companies interested in antibiotic development focused more on broad-spectrum agents to widen the potential market. Second, there has been an overall drop in approvals for new antibiotics over the last 30 years (Fig. 1), which is concerning since resistance to available drugs is rapidly climbing leading to some experts calling this a "perfect storm".[23–26] However, this drop in approvals may not be as dire as it initially appears. Only looking at the trend in the number of approvals conceals the fact that many of the antibiotics released in the 1980s and 1990s were perhaps rushed through development too quickly and have since been

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discontinued while newer drugs have thus far fared much better (Fig. 1). Many of the second and third generation cephalosporins approved in the 1980s had overlapping clinical utility with better selling family members and were withdrawn due to poor sales, while several fluoroquinolones approved in the 1990's were withdrawn for safety reasons.[27] Despite the declining trend in antibiotic approvals, 2014 witnessed an uptick with four new molecular entities receiving FDA approval (1. dalbavancin, 2. oritavancin; both vancomycin derivatives, 3. ceftolozane; a 5th generation cephalosporin, and 4. tedizolid; an oxazolidinone related to linezolid).

The uptick of new approvals may have been spurred in part by a heightened academic and media interest in antibiotic development and conservation as a response to the dire consequences of antibiotic ineffectiveness. More recently, government organizations have been promoting antibiotic discovery as well. The United States congress passed the Generating Antibiotic Incentives Now (GAIN) Act in 2012 to financially incentivize antibiotic development[28,29] followed by Executive Order 13676 (Combating Antibiotic-Resistant Bacteria), which directs government agencies to promote the development of new drugs and diagnostics, identify means of slowing resistance, and strengthen surveillance efforts for resistant bacteria.[30,31] A number of specific suggestions have been put forward to stimulate antibiotic development, including providing further financial incentives like research and development tax credits and grants, easing regulatory constraints, and promoting industry-academic collaborations.[23,32,33] New ideas to identify novel antibiotics are also appearing in the literature. Given previous successes with natural products serving as antibiotic lead structures,[34] strategies promoting natural product discovery with new cultivation techniques and molecular methods to reconstitute or activate specific gene clusters are especially promising.[15,35–42] Another potentially successful approach is reexamining old drug leads that were previously abandoned during development, often because they were not considered broad-spectrum enough at the time.[43] Repurposing drugs designed for other indications can also produce new antibiotics or anti-virulence agents, with the added benefit of known safety and pharmacokinetic profiles.[44,45]

Expanding the focus of antibiotic development to narrower spectrum compounds opens the door to the development of previously discarded leads, and makes it easier to find new ones by removing the requirement that a compound is growth inhibitory towards evolutionarily diverse bacterial pathogens.[15] Additional benefits associated with narrower spectrum drugs range from the possibility of slower resistance generation[46–48] to the demonstrated decreased risk of antibiotic-associated colitis (i.e. *Clostridium difficile* infections).[49–51] However, efficacious use of narrow-spectrum antibiotics requires rapid and sensitive diagnostics to identify the bacterial cause of infection. The current gold standard diagnostic is still traditional culture-based clinical microbiology, which is slow and often insensitive, such as with blood culture for sepsis patients.[52–55] Furthermore, determination of an antibiotic resistance profile adds even more time to this process. Ironically, the ready-availability of numerous, effective broad-spectrum drugs that saved so many lives also contributed to a stagnation in diagnostic advances for decades.[56] Only in the past 15 years have improved diagnostic techniques begun to gain momentum. The introduction of new molecular techniques, especially those that are polymerase chain reaction (PCR)- and mass spectrometry (MS)-based, have primarily driven this innovation and, along with several as

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yet clinically unevaluated technologies, have already resulted in tests that shave hours or even days off traditional diagnostic methods.[57]

Looking to the future, narrow-spectrum antibiotics and rapid diagnostic tests will ideally co-evolve. The effectiveness of this strategy has already been demonstrated in cancer therapy with companion diagnostics[58] and we argue that a similar effort is needed for bacterial infections. Employing rapid diagnostics can provide multiple benefits to the patient and to hospitals but coupling such tests to an antibiotic is currently impractical due to the lack of pathogen-specific drugs. Conversely, pathogen-specific drugs will be ineffective without the development of rapid and reliable diagnostics. Thus, this review will first focus on trends and issues that must be addressed in narrow-spectrum antibiotic discovery and use. The second portion will then examine the advances in diagnostics and discuss future needs, with an emphasis on what is required to effectively utilize new narrow-spectrum agents.

2. Narrow-spectrum antibiotics

2.1. Definition and therapeutic benefits

As a preface to a discussion on the benefits and drawbacks of antibiotics with different spectra of activity, it is important to define what exactly is meant by the terms “narrow-spectrum” and “broad-spectrum”. The terms were introduced in the 1950’s as comparators to describe the obvious differences between the original penicillins, such as penicillin G, and the broader spectrum tetracyclines and chloramphenicol.[59] When used to compare two antibiotics with different spectra of activity, broad- and narrow-spectrum are relatively easy to define. However, the terms have evolved over time into sweeping categories into which compounds with very different activities are lumped.[59] This has resulted in discrepancies in the literature wherein an antibiotic described as broad-spectrum in one paper may be called narrow-spectrum in another, often with no explanation as to why the label was chosen in either case. The phenotypic Gram stain is also frequently used as part of descriptions of narrow-spectrum antibiotics; for instance, many agents act selectively on Gram-positive bacteria because the drug cannot penetrate the outer membrane of a Gram-negative organism. However, this can be misleading in the case of organisms that lack an outer membrane but are still phenotypically Gram-negative (e.g. mycobacteria).[60] Additionally, many antibiotics that target only Gram-positive or Gram-negative species are still broadly active within that category. In this review, we will reserve the term narrow-spectrum for antibiotics that have a reasonable likelihood of only affecting one or a small handful of species when administered to a patient, either through specifically targeting a single species or by targeting virulence factors that would be generally absent from non-pathogenic bacteria. However, we include a discussion of antibiotics that are selective for a single Gram stain phenotype as these are often considered to be narrow-spectrum[61,62] and are certainly narrower spectrum than many other available agents.

Historically, the development of broader spectrum drugs by synthetically modifying existing antibiotics was the goal of pharmaceutical companies.[4] This can be readily seen with beta-lactam antibiotics, which were designed to achieve broader activity with the second and third generation compounds.[63] This approach is logical from a number of standpoints, most notably offering pharmaceutical companies the highest potential return on investment, as the

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drug could theoretically be used for multiple types of infections. Furthermore, broad-spectrum drugs allow doctors to treat infections empirically with a higher likelihood of success.[64] This is critical in life-threatening situations and broad-spectrum antibiotics will likely always have a place in medicine for this purpose.[64] The simplicity of the "one drug treats all" approach is appealing, but retrospectively we can no longer ignore antibiotic resistance and other associated problems like *C. difficile* infections. While a number of factors contribute to the rate at which resistance appears and spreads, including the frequency of use and misuse, the type of resistance mechanism required, and the fitness of resistant organisms,[65,66] the use of broad-spectrum antibiotics appears to be correlated with increased emergence of resistance.[46–48,67] Accumulation of resistance to antibiotics other than the one employed in treatment has been observed as well,[47] resulting from horizontal gene transfer (HGT) of DNA cassettes containing multiple resistance genes.[68–70] Administration of several broad-spectrum antibiotics, especially those that accumulate in the intestines, can have a devastating impact on the microbiome that persists for months or even years after cessation of treatment.[71–74] As alluded to in section 1, disturbances of the gut microbiome can lead to a number of issues, most notably *C. difficile* infections.[75,76] *C. difficile* thrives in the human colon when other bacteria are not present to suppress its colonization and growth.[77,78] Secondary infections like those caused by *C. difficile* are directly linked to antibiotic usage, especially broad-spectrum cephalosporins, fluoroquinolones, and clindamycin.[75] Thus, *C. difficile* is difficult to eradicate with antibiotics owing to a catch-22-like scenario. As such, healthcare-associated strains of *C. difficile* have quickly become a global problem.[79] Additionally, antibiotics may be contributing to other issues linked to changes in the gut microbiome including chronic inflammatory diseases, diabetes, and asthma.[80–84] It is becoming increasingly obvious that the health of the microbiome must be taken into consideration during the development of future antibiotics.

Due to the drawbacks of broad-spectrum antibiotic usage and the general difficulties associated with finding new drugs capable of inhibiting the growth of a range of evolutionarily diverse bacterial pathogens, there have been calls in the literature to give renewed consideration to narrow-spectrum compounds.[56,85] A number of possible benefits to using narrower spectrum drugs have been postulated, including those mentioned above, although some of the benefits are either theoretical, anecdotal, or are based on limited clinical data. In contrast to broad-spectrum treatment, the use of narrow-spectrum drugs may slow the spread of resistance through a lessened impact on the human microbiome, leading to reduced HGT of pre-existing resistance mechanisms.[48,74,86] Further studies are needed to confirm the trend, but large scale correlative evidence of this can be seen in Europe, where northern European countries tend to prescribe narrow-spectrum antibiotics more frequently than their southern neighbors on a proportional basis.[86–88] However, it is difficult to determine the direct impact of narrow- versus broad-spectrum antibiotic usage on a large scale since resistance rates also correlate heavily with frequency of usage. Countries that take a narrower spectrum approach also tend to be more conservative towards antibiotic usage overall and an increase in use with possible new narrow-spectrum antibiotics could still potentially cause a rapid rise in resistance. Sparing the gut microbiome has also been shown to correlate with a lower rate of childhood obesity as compared with the use of broad-

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spectrum drugs.[89] Although direct evidence for the impact of broad- versus narrow-spectrum antibiotics on other conditions associated with the human microbiome is scarce, it is not difficult to imagine that significant links exist. As for the treatment efficacy, numerous studies have demonstrated that narrow-spectrum drugs can be just as effective as broad-spectrum ones in certain circumstances, especially in prophylaxis.[90–93]

Narrow-spectrum drugs have been developed and marketed since the advent of antibiotics, starting with salvarsan for syphilis. However, there has been a recent uptick in FDA approvals of antibiotics with narrower spectrums of activity, especially drugs targeting Gram-positive bacteria (Fig. 1). Pathogen-specific antibiotics that target only one or a small set of species are also receiving increased interest, with fidaxomicin being recently approved as a selective agent for *C. difficile* that permits the gut microbiome to recover.[74,94] Other strategies for fighting bacterial infections, such as targeting virulence[3,95,96] or treatment with antibodies or phage,[56,97] are alternatives to growth-suppressive, small molecule antibiotics that spare the microbiome and possibly slow resistance. However, these approaches must be demonstrated to cure patients as effectively as traditional antibiotics or they will not gain FDA approval, let alone find clinical utility. Even if treatment with a broad-spectrum antibiotic leads to the spread of resistance in the longer-term, the immediate need of the patient will likely outweigh what may be best for the community at large.[98,99]

2.2. Antibiotics selective for one Gram stain group

Until recently, narrow-spectrum generally meant that an antibiotic was only considered to be active against either Gram-positive or Gram-negative bacteria, with the exception of drugs for tuberculosis (TB) and salvarsan for syphilis. However, as noted in section 2.1, many of these drugs are still rather broad-spectrum within one Gram stain grouping. Many of the early antibiotics are Gram selective, but this shifted to a trend of even broader spectrum compounds in the 1980s and 1990s as medicinal chemists continuously tinkered with the properties of existing drugs.[4] This resulted in the release of ever more broad-spectrum fluoroquinolones and second and third generation cephalosporins during that time period.[34] More recently, there has been a shift towards the approval of more antibiotics active only against Gram-positive bacteria (Fig. 1), spurred in part by the prevalence and threat posed by multiple drug-resistant (MDR) *S. aureus* and *Streptococcus pneumonia*. These two pathogens alone were responsible for over 75% of deaths from antibiotic-resistant infections in the United States in 2013.[8] Discovering compounds with Gram-positive-only activity is theoretically easier than for Gram-negative activity, since the outer membrane present in most Gram-negative bacteria presents a formidable barrier.[100,101] Focusing on a subset of bacterial species helps circumvent problems posed by the diversity among species that must be considered when attempting to develop broad-spectrum antibiotics,[102] though targeting an entire Gram stain grouping may not be specific enough to reap the potential benefits of narrow-spectrum agents.

Two notable examples of the renewed interest in narrowing the spectrum of antibiotics by targeting Gram-positive pathogens are the oxazolidinone and lipopeptide classes (exemplified by the first-in-class agents linezolid and daptomycin, respectively) (Fig. 2).[102] Intriguingly, both compounds had been identified as potential leads, but discarded because

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of toxicity concerns.[103–105] However, in both cases, different pharmaceutical companies picked up the development of the compounds, overcame the toxicity issues, and brought them to market.[103,105] Although both drugs are still broad-spectrum enough to be clinically useful in a number of infections, the fact that both have achieved blockbuster status is a testament to the potential for financial success with emerging narrow-spectrum antibiotics. A number of other Gram-positive selective antibiotics have been successfully released since, including glycopeptide and oxazolidinone family members.

Despite clinical and financial success, drugs targeting a single Gram stain grouping share the limitations of broader spectrum antibiotics. For example, the clinical appearance of resistance to linezolid and daptomycin was not significantly slower than for other antibiotic classes.[5,6] This may partly be a consequence of the success of the drugs, with high usage leading both to blockbuster status and the rapid development and dissemination of resistance. In theory, narrower spectrum antibiotics should have a reduced impact on the human microbiome, although this does not always appear to be the case for Gram selective drugs. Oral vancomycin treatment, although rare and generally reserved for *C. difficile*-associated diarrhea/colitis,[74] induces dramatic changes in the gut microbiome similar to broader spectrum antibiotics.[72,106,107] Indeed, orally administered vancomycin kills *Bacteroides* species that are not susceptible *in vitro*[108] which has been attributed to vancomycin concentrating to unusually high levels in the gut due to poor oral absorption;[109,110] however, this may also stem from the knock-on effect of wiping out a subset of the microbiome that produces factors that other members rely on for stable colonization.[74] The latter possibility is potentially an issue for all narrow-spectrum antibiotics that affect even a single off-target species.[106,111] Moving forward, the impact of Gram selective versus broader spectrum drugs on the human microbiome, as well as on resistance, will need to be examined in detail on a compound-by-compound basis to determine if they do provide an advantage.

2.3. Pathogen-specific antibiotics

In contrast to Gram selective antibiotics, pathogen-specific narrow-spectrum antibiotics would theoretically be used against a specific pathogenic species/genus rather than a general disease category like sepsis or urinary tract infections (UTI). This would constitute a type of personalized medicine with clear parallels to newer cancer drugs that are marketed with companion diagnostics. In cancer therapy, the diagnostic serves the purpose of determining whether the genotype of the patient's cancer cells is a match for the drug, while with an infectious disease the specific invading pathogen would be identified.[58,112] The main advantage of such a personalized strategy is that treatment would be expected to minimize collateral damage to the microbiome[94,106,111] and perhaps even delay resistance acquisition by HGT. Pathogen-specific drugs have been used for decades in the treatment TB, with a number of FDA-approved drugs that are only active against *Mycobacterium tuberculosis* and other related mycobacteria (isoniazid, ethambutol, etc.).[113] The focus on TB stems in part from the staggering number of people infected (an estimated 2 billion worldwide) and the difficulty in treating the disease (6 months with multiple drugs).[113] While mycobacteria-specific antibiotics are effective at treating TB, a cocktail of several drugs that usually includes broad-spectrum antibiotics as well is necessary to prevent the rapid generation of

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resistance.[113] Thus, TB presents a well-studied counter-point to the idea that pathogen-specific antibiotics may help to slow the spread of resistance. Nonetheless, the other potential advantages to pathogen-specific treatment discussed in section 2.1 may still be beneficial, especially given the extended duration of treatment.[113] Since TB does not kill quickly in most cases, there is time to accurately diagnose the disease through culture-based methods and begin a treatment regimen that includes TB-specific antibiotics. It should be noted though, that the existence of TB-specific drugs certainly had more to do with clinical efficacy rather than with a conscientious attempt to spare the microbiome. Mycobacteria contain a number of unique targets related to the cell wall that facilitated the development of specific antibiotics.[114] Generalizing beyond TB selective drugs, there is no reason to think that pathogen-specific antibiotics couldn't be developed for other pathogens as well, although it is likely that differentiating between two Gram-negative species, for example, would be more difficult than differentiating between mycobacteria and other pathogens. Very narrow-spectrum antibiotics are anticipated to be useful primarily for mono-microbial infections, and only then if the infecting pathogen can be rapidly and accurately identified. Identification would either be through standard clinical microbiology or the observation of symptoms unique to a pathogen. If multiple antibiotics targeting a specific pathogen could be developed, they could be used as part of a cocktail therapy to help extend the lifetime of the drugs, similar to the treatment regimens for TB.

Partly because the pharmaceutical industry preferentially develops (and doctors prescribe) the most broad-spectrum agents possible, the targets of current antibiotics are ubiquitous in the domain bacteria. Antibiotics that target a single Gram stain grouping share the same targets and are only selective due to the presence of other cellular features like the outer membrane of most Gram-negatives and efflux pumps that prevent the accumulation of therapeutic concentrations inside the cell. To develop an antibiotic against a specific species, unique targets that are not only essential to cell survival but also do not exist or can be compensated for in other bacteria are required. The presence or absence of important enzymes, unique cellular components, and key metabolic pathways are possible future drug targets. Additionally, specific protein folds or 3D structures unique to a single species in the otherwise common targets of other antibiotics could also be investigated. Prodrugs that are only activated once taken up by a specific pathogen would also impart specificity to compounds that become toxic when metabolized, such as with isoniazid in the treatment of TB.[115] Designing antibiotics around a specific target in one pathogen may even prove to be advantageous in that drug leads would not be required to have activity against distantly related homologs in other species. However, targets would need to be chosen carefully to ensure that sufficient differences between homologs exist and it remains to be seen if this is going to be a viable strategy.

A bountiful source of pathogen-specific drug leads may remain to be discovered in the realm of natural products. Some of these yet-to-be-discovered compounds may specifically interact with ecologically neighboring species while leaving others unaffected, either as signaling molecules or as mechanisms to fend off competitors. A compound that acts as a signaling molecule for a specific species at environmentally relevant concentrations may kill the recipient outright while still maintaining specificity at higher concentrations. Even in the absence of co-evolution of species, microbial natural products are endowed with properties

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that render them better able to enter bacterial cells and interact with efficacious target(s).[116,117] In addition to whole-cell screening with new compounds, pathogen-specific antibiotic discovery can also occur through screening against unique targets either *in vitro* or *in silico*, though serious pitfalls exist for these methods as discussed in section 1 and reviewed by Payne et al.[16] To date, few pathogen-specific antibiotics have been reported and still fewer have been followed up on to any significant extent. The scarcity stems in part from the limited antibiotic testing performed on many new natural products. Often, only common pathogens such as *S. aureus* and *Escherichia coli* are screened during testing so select activity against rarer pathogens is undetected (although the financial realities of developing such a compound would likely be limiting). Conversely, if only a small handful of organisms are used, a compound with activity against only one tested strain may also have activity against other untested pathogens or against the multitude of species in the human microbiome. As always, the researcher can only detect what he/she screens for.

Outside of TB-specific drugs, the only pathogen-specific antibiotic in use with FDA approval is fidaxomicin for the treatment of *C. difficile* (Fig. 3),[94] which is ironic given that the explosion in *C. difficile* cases is the direct consequence of widespread use of broad-spectrum antibiotics. Although fidaxomicin does display some activity against other species, the minimum inhibitory concentrations in these cases tends to be 10–100 fold higher than for *C. difficile*,[118] allowing for the specific treatment of that pathogen with minimal effects on the gut microbiome.[74] Fidaxomicin has been shown to reduce recurrence rates of *C. difficile* in comparison to vancomycin, likely by allowing the gut microbiome to recover.[119,120] The deployment of fidaxomicin to treat *C. difficile* infections represents an important milestone; however, the cure rates are still significantly lower than those achieved by fecal transplant.[120,121]

A number of other pathogen-specific antibiotics have been reported in the literature but have not yet found clinical use. The following examples are not meant to be an exhaustive list of pathogen-specific agents and additional examples can be found in other reviews.[122,123] Microcins are ribosomal peptide antibiotics, some of which are extensively post-translationally modified and display exquisitely selective activity.[124] A notable example is microcin B17 (Fig. 4), exerting activity against only a handful of related gamma-proteobacteria that lack the associated immunity gene that protects the producing strains.[124,125] The microcin B17 peptidic framework is decorated with a number of side chain-derived thiazole and oxazole rings[124] that rigidify the conformation of the peptide and provide an interaction surface to inhibit DNA gyrase.[126,127] While microcin B17 does not exhibit the type of small molecule structure typically associated as being “drug-like”, other peptides (and even larger biologics) have found clinical utility, such as the HIV fusion inhibitor enfuvirtide (Fuzeon) or insulin for diabetes.[128] Several other ribosomal peptides have been recently reported with pathogen-specific activity including the natural product plantazolicin (Fig. 4), which, as another thiazole/oxazole-containing peptide, is biosynthesized in a similar manner to microcin B17 by *Bacillus* a*myloliquefaciens* and *Bacillus pumilus*.[129,130] Plantazolicin has selective activity against *Bacillus anthracis* but the biological target has not yet been reported.[131,132] The semisynthetic lanthipeptide NVB302 (from the natural product deoxyactagardine B) is another example of a ribosomal peptide-derived, pathogen-specific antibiotic (Fig. 4);[133] it has recently completed a phase I clinical

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trial for the selective treatment of *C. difficile*.[134] Looking to more traditional, small molecule antibiotics, promysalin (Fig. 4) is a novel type of amphipathic salicylic acid-containing antibiotic produced by *Pseudomonas putida* with exquisitely selective activity against only other members of the *Pseudomonas* genus including *P. aeruginosa*, an ESKAPE pathogen.[135,136] Yet another example is the pyloricidins (Fig. 4), a family of peptide-like small molecules produced by *Bacillus* sp. HC-70 and HC-72.[137,138] These compounds were discovered by screening specifically for activity against *Helicobacter pylori*, a traditionally difficult to treat gastric pathogen.[139] Like the example provided by *M. tuberculosis*, the selective targeting of other traditionally difficult pathogens may be the ultimate niche for further pathogen-specific antibiotic development.

Most of the above examples demonstrate selectivity and potency *in vitro* which does not necessarily mean they would translate well into drugs. Additional studies are required, including determination of safety and pharmacokinetics, and the compounds (or derivatives) would need to meet all the stringent requirements for development into a drug. Whether this will happen is questionable given the high cost of drug development and the relatively small market for an antibiotic specific against rarer pathogens. A serious impediment in the development of pathogen-specific antibiotics is recruitment of sufficient patient populations known to be infected with the targeted pathogen, adding complexity to the clinical trials. Granting orphan drug status to these compounds may help but additional financial incentives would likely also be required for pharmaceutical companies to proceed with development. Governmental agencies may have an interest in providing incentives for pathogens of homeland security concern, like *B. anthracis*. In the absence of subsidies however, it is probable that only pathogen-specific antibiotics against ubiquitous pathogens like methicillin-resistant *S. aureus* (MRSA) will attract interest from industry, analogous to the case of fidaxomicin for *C. difficile*.

2.4. Anti-virulence agents

An alternative strategy for treating disease caused by a particular bacterial pathogen is to interfere directly with pathogenesis. Such an anti-virulence strategy is anticipated to reduce the pathogen's ability to cause disease, rather than be growth suppressive. This entails targeting molecular entities that are not essential for the survival of the pathogen *in vitro* but are required for invasion and/or survival in the host.[3,95,96] A number of virulence targets have been explored, including inhibition or over-activation of quorum sensing,[140,141] inhibition of bacterial adhesion,[142,143] and inhibition of toxin production or delivery.[95] Since virulence factors are often unique to a single or small set of pathogens, agents that target them will intrinsically be pathogen-specific, although there are certainly cases in which members of the microbiome have similar factors that help them stably colonize a host without causing disease.[144] Targeting virulence rather than viability has also been postulated to cause slower resistance development due to the fact that the agent is not growth suppressive and thus elicits less selective pressure for acquiring resistance.[3,95,145] However, this prediction has not been sufficiently investigated and recent studies involving quorum sensing inhibition have cast some doubt on the idea.[146] We postulate that the propensity to develop resistance with anti-virulence agents will depend strongly on a number of factors, including how critical the virulence factor is to the pathogen for maintaining fitness in the

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host. If the virulence factor is essential to survival in the host, one would expect resistance to rise at approximately the same rate as if the cell wall or the ribosome were being targeted. Although it remains to be tested, anti-virulence agents also may not deliver clinical efficacy if supplied to patients as a monotherapy.[85] Formulating anti-virulence agents with immune-stimulating drugs,[147,148] or even a more conventional antibiotic, could prove to be the best future strategy for the patient and for the community.

A number of compounds that interfere with virulence through a wide array of pathways have been reported in the last decade. One of the first notable examples was virstatin (Fig. 5).[149] It was found to transcriptionally prevent cholera toxin and pili production in *Vibrio cholerae* without causing any growth effects.[149] An *in vivo* infection model in mice demonstrated that virstatin treatment achieved a significant decrease in the bacterial burden.[149] Interestingly, virstatin was also recently found to inhibit biofilm formation in the ESKAPE pathogen *A. baumannii* through inhibition of pili biogenesis.[150] Another example of an anti-virulence agent that showed efficacy *in vivo* is BPH-652 (Fig. 5), which was originally designed to inhibit cholesterol biosynthesis by targeting human squalene synthase. BPH-652 was also found to block a homologous enzyme in *S. aureus*, dehydrosqualene synthase, leading to inhibition of the oxidative stress-protective pigment staphyloxanthin.[151] Without the protection of staphyloxanthin, *S. aureus* was much more susceptible to the reactive oxygen species delivered by host immune cells and was cleared more efficiently in a mouse model of infection.[151] In addition to compounds that inhibit pathogenesis, there have been efforts to develop strategies to counteract toxins such as those produced by *B. anthracis* and *Clostridium botulinum*,[152,153] similar to existing antibody based anti-toxin therapies (e.g. raxibacumab for anthrax toxin).[154] These types of compounds would not necessarily prevent or clear an infection alone but would rather reduce the damaging effects of the toxins and increase the chances of patient recovery. This is particular important for the pathogenic mechanism of diseases like anthrax and botulinum, which can cause mortality even if the causative pathogen is eradicated.[155,156] It may also be possible to develop pseudo broad-spectrum anti-virulence antibiotics by purposefully targeting virulence factors that are employed by multiple different pathogens, such as the streptolysin S family of cytolytic toxins.[157] We found that the HIV protease inhibitor nelfinavir (Fig. 5) blocked the proteolytic maturation of streptolysin S in *Streptococcus pyogenes* as well as related cytolysins from other pathogenic Firmicutes.[158] Such an approach may addresses the niche marketability issue associated with pathogen-specific antibiotics while still providing the benefit of not disturbing the human microbiome.

Despite the potential benefits of anti-virulence antibiotics, the fact that they don't suppress growth directly could also prove to be a major pitfall. Some pathogens deploy large arsenals of virulence factors[159–161] and inhibiting a single one may not sufficiently reduce their pathogenicity. Not all virulence factors are important throughout the course of infection either, with some required only for initial invasion or for dissemination to other body sites. This could prove to be an issue if treatment isn't started until symptoms appear and the infection is well underway, although utilization in prophylaxis for immune compromised individuals or surgery patients could still be helpful. A further complication is that many pathogens have variant strains that don't produce specific virulence factors and yet still cause disease; virulent strains of *V. cholerae* that do not produce cholera toxin and non-

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hemolytic strains of *S. pyogenes* that lack streptolysin S are examples of this.[149,162,163] If advances in diagnostics can overcome these challenges, however, anti-virulence agents could be an excellent avenue for treating bacterial infectious disease. Additionally, coupling anti-virulence drugs with conventional agents in a multicomponent cocktail, as is done in HIV, cancer, and TB therapy, could serve to significantly improve patient outcomes by directly reducing bacterial counts with the survivors more vulnerable to the host's immune system.

2.5. Alternative narrow-spectrum therapeutic approaches

Another unconventional method for designing narrow-spectrum antibiotics is to attach broad-spectrum antibiotics, or even broadly toxic compounds, to a narrowly specific targeting domain.[164] This strategy has been employed in cancer therapy with several FDA-approved antibody-drug conjugates [brentuximab vedotin (Adcetris), trastuzumab emtansine (Kadcyla)].[165] Examples of targeting domains include peptides,[166,167] antibodies,[168] and phage.[169] The conjugates are intended to bind specifically to the pathogen of interest and thus create a locally high concentration of the antibiotic that is sufficient to kill the target. This strategy theoretically prevents non-targeted bacteria (and host cells in the case of general toxins) from experiencing harmful concentrations of the toxic payload. Oral administration of these antibiotic conjugates would likely be infeasible due to stability and bioavailability issues. The requirement of successfully targeting the pathogen and then delivering a sufficiently toxic payload also presents two different routes for resistance generation. Attempting to fight the pathogen outright with antibodies or bacteriophage may be more realistic. Both strategies have historical precedent and have been reviewed extensively elsewhere.[170–172] The use of bacteriophage to deliver CRISPR-Cas (CRISPR, clustered, regularly interspaced, short palindromic repeats; Cas, CRISPR-associated protein) systems for selective bacterial killing is also beginning to be explored.[173] Cas proteins are a nucleases capable of cleaving DNA in a site-specific manner dependent on targeting by ~20–40 nucleotide RNA guides (CRISPR units).[174,175] Cleavage of the bacterial genome by Cas nucleases has been shown to be lethal[176] and antimicrobials utilizing this strategy are currently in development by Eligo Bioscience.

2.6. Challenges facing narrow-spectrum therapy

If narrow-spectrum antibiotics manage to overcome the technological and financial hurdles to their development, they still face significant challenges in the clinic. An accurate and sensitive diagnosis would be required to rule out the possibility of a polymicrobial infection, otherwise a patient's condition may only worsen as an undetected pathogen flourishes during treatment of the known one. Additionally, immunocompromised patients are more susceptible to secondary infections that could be suppressed by a prophylactic broad-spectrum antibiotic but would be unaffected by a narrow-spectrum agent.

One of the intended benefits of narrow-spectrum antibiotics is that they would be less likely to disturb the human microbiome. However, there are an estimated 800 or more species in the gut[177] and new antibiotics are unlikely to be tested against a vast majority of these, especially given the difficulty in culturing many species in the microbiome. Even a pathogen-specific antibiotic may have unanticipated effects on some of these species, which could end up impacting the entire microbiome with deleterious effects. The argument that

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resistance may be slower to develop due to less possibility for HGT also loses some of its relevance if this is the case. The spectrum of activity of antibiotics against the gut microbiome could be tested indirectly through metagenome analysis of fecal samples however, and this approach would also provide a more realistic picture of the effect of narrow-spectrum antibiotics in the context of the host. Regardless, resistance will always develop as bacteria eventually find a way to survive. Given the difficulty in finding novel antibiotics, strategies that provide new avenues to discovery or slow the spread of resistance are welcome and will hopefully be successfully combined in the future with other methods of fighting infections, such as boosting the human immune system or improved sterilization and sanitation in hospitals.

3. Diagnostic techniques

3.1. The need for advanced diagnostics

To best utilize narrow-spectrum antibiotics, it is imperative to know which organism is causing the infection. In some cases, the manifestation of disease is indicative of a specific pathogen, such as an erythema migrans rash with Lyme disease, and any future narrow-spectrum antibiotic could likely be prescribed before further testing. Similarly, around 80% of UTI cases are caused by *E. coli*[178] and, since they are generally non-life threatening, could be initially treated with an *E. coli*-specific antibiotic until diagnostic testing indicates a different pathogen is present. Narrow-spectrum antibiotics could also be used for prophylaxis, targeting the pathogens most often responsible for complications following surgeries or implants. However, most diseases can be caused by a multitude of different pathogens and thus the need for rapid and accurate diagnosis inherently goes hand in hand with the use of narrow-spectrum antibiotics.

Traditional culture-based diagnostic methods combined with Gram staining can take 1–2 days to identify a pathogen, or even longer for slow growing organisms.[54,55,179] Subsequent determination of resistance by phenotypic methods (Kirby-Bauer testing, microbroth dilutions, Etests) then requires up to an additional day before results are available.[180,181] This extended time frame results in patients being treated empirically with broad-spectrum agents that may or may not be de-escalated when appropriate after testing is complete. After this delay, the advantage of treating with a narrow-spectrum antibiotic may be lost, with the microbiome already perturbed and potentially problematic organisms on the rise. The sensitivity and specificity of some gold standards leaves something to be desired as well, with blood culture failing to detect pathogens up to 50% of the time after empiric treatment has started[52,182] and sputum samples often resulting in the growth of multiple species in culture.[179]

To sufficiently improve upon traditional methods such that narrow-spectrum antibiotics can be readily employed, several important requirements must be met (Table 1). Specifically focusing on diagnostics for narrow-spectrum antibiotic use, the most important criteria are the time to result and the accuracy and sensitivity of the test, especially with regards to the ability to rule out polymicrobial infections. Ideally, a point-of-care diagnostic test would be rapid enough to provide a result in time for the initial treatment decision. For outpatient care, this would ideally occur during the visit. For inpatients, especially those in critical condition,

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getting results as soon as possible is essential.[183,184] Reducing delays in appropriate antibiotic treatment for critical conditions such as sepsis has been repeatedly shown to have a major impact on patient survival.[184–186] Fulfilling this speed requirement will likely mean development of devices that are portable or can be set up in hospital clinical microbiology labs, as sending samples to a third party would cause a significant delay.

The other central requirements of high accuracy and sensitivity for diagnostic tests are particularly important for pathogen-specific antibiotics. Misidentification or the failure to detect a pathogen would lead to an incorrect treatment strategy with potentially dire consequences.[184] Identification of polymicrobial infections would also be crucial to prevent treatment failure due to the second, untreated pathogen.[187,188] Many newer diagnostic methods are designed around technologies that simply do not work well when multiple species are present in the sample.[189,190] False negatives with rarer pathogens resulting from molecular diagnostics that only test for the most common causes of infection is an additional concern, although this certainly applies to treating with any antibiotic regardless of spectrum. Thus, tests that can incorporate the detection of the largest number of potential pathogens while maintaining a high degree of specificity and sensitivity are warranted.

Beyond speed and accuracy, several other factors should be considered when comparing new diagnostic technologies.[55] Tests that can incorporate rapid resistance determination have a significant advantage in utility, especially if high levels of resistance are known to exist for the narrow-spectrum antibiotic that could be employed. From a financial standpoint, expensive instrumentation may be prohibitive for smaller institutions while a high cost per sample may discourage routine use, particularly if the test is not readily accepted for reimbursement by insurers. Another consideration is the amount of hands on time required to perform the test. A test that only takes an hour to complete but requires the attention of a technician the entire time for a single sample may not be feasible. More sample manipulation also leads to more opportunities for contamination, leading to inaccurate results. Finally, tests that provide quantification may be useful for the differentiation of colonizing and infectious organisms although the benefit of this has been called into question.[191]

While improved diagnostics are discussed here in terms of their ability to aid in narrow-spectrum antibiotic use, it is important to keep in mind that there are other advantages to improved diagnostics such as tracking outbreaks and epidemics, monitoring resistance, and discontinuing isolation of patients falsely suspected of having highly infectious diseases. We will first review the two main areas of advancement in diagnostics, nucleic acid- and MS-based methods. Many of the techniques from these two methods have been extensively tested and have already begun to enter the clinic. We will then briefly touch on emerging technologies that have the potential to significantly improve diagnostic methods in the future.

3.2. Nucleic acid-based technologies

New molecular biology techniques developed over the last few decades were quickly seized upon by the diagnostics field for novel strategies for pathogen identification. Nucleic acid-based technologies (NATs) utilize bacterial DNA or RNA to rapidly pinpoint the infecting

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pathogen either through hybridization leading directly to a signal or through the amplification of DNA. Both strategies have been extensively developed with commercial tests available that offer significant improvements over traditional culture-based methods.

3.2.1. Non-amplification NATs—The field of non-amplification based NATs is primarily focused on peptide nucleic acid (PNA) fluorescence in situ hybridization (FISH) techniques. PNA-FISH based tests were among the first new molecular diagnostics to receive FDA clearance and have been available for clinical use since the early 2000s.[192] The tests report the presence of the target bacteria via a fluorescent signal generated upon hybridization of a short PNA probe (<25 bases) to bacterial RNA or DNA.[193] Due to its high cellular abundance and sequence differences between organisms in the variable regions, ribosomal RNA (rRNA) is often used as the target for PNA probes.[194] The use of PNA probes instead of DNA minimizes electrostatic repulsion, allowing for rapid and tight binding.[195] Short PNA probes are also able to cross the cell membrane, removing any need for a lysis step and reducing hands on time.[196] Extremely rapid commercial kits are available that can provide results in under an hour (e.g. *Quick*FISH, AdvanDx)[197] and it has been demonstrated that PNA-FISH can provide excellent accuracy in a clinical setting.[198,199] However, PNA-FISH methods require a high bacterial count for detection and have thus far only been designed for use on positive blood cultures, resulting in a 1–2 day delay from when a patient sample is collected to when the test can be performed.[192] While this is a significant improvement on identification of pathogens by subculturing from a positive blood culture, the sensitivity of the method would need to be dramatically improved to allow the direct testing of patient samples required for a truly rapid result. Additionally, since rRNA is targeted, the presence of resistance markers is not analyzed at all with current methods and an amplification step would likely be required to detect the relatively low abundance of RNA from resistance genes. Like most NAT methods, PNA-FISH requires a specific probe or set of probes for each target organism. This results in an increasing cost per sample as additional probes for other pathogens are included, although the initial instrumentation costs are minimal.[192] As PNA-FISH does not multiplex (simultaneously measure multiple analytes in a single assay) effectively and will miss the presence of any pathogens not specifically targeted, it is not ideally suited for diagnosis prior to narrow-spectrum antibiotic use.

Several other promising hybridization techniques have also been proposed for bacterial detection and identification, although these have not been nearly as extensively validated as PNA-FISH. One such technology is NanoString, an RNA detection method that is commercially available with the nCounter system.[200] NanoString allows for bacterial detection through the use of capture and reporter probes specific to targeted RNA sequences, with the reporter probe containing sets of colored indicators that are read through microscopy.[200,201] The technique has been shown to be effective for bacterial, viral, and fungal pathogen detection with the use of several probe pairs per species.[201,202] Up to 800 probes can be multiplexed in a single reaction, allowing a large number of species to be targeted at once.[200] However, the upfront cost of the instrumentation is in the hundreds of thousands of dollars and the technique requires a long hybridization time (>12 h),[200]

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although a recent report shortened this significantly, allowing the entire technique to be performed in around 8 h.[203]

An emerging technique for RNA detection via hybridization is the use of microring resonators. Microring resonators detect changes in the local refractive index upon binding of target molecules.[204] By functionalizing the rings with DNA capture probes designed for species specificity, bacterial detection based on target RNA molecules can be achieved in <1 h after RNA isolation.[205–207] Microring resonators can be inexpensively manufactured with many rings on a single, small chip, allowing for highly multiplexed detection of a large number of targets.[205] Both microring resonators and NanoString allow for a degree of quantification,[200,206] which, unlike PNA-FISH, provides the advantage of possible resistance determination by examining transcriptome changes during antibiotic treatment in susceptible organisms.[201] Despite the ease of multiplexing these two technologies, untargeted organisms will still be missed resulting in rarer pathogens evading detection. Additionally, both methods have only been tested with relatively high bacterial counts so the limits of detection may not be sufficient for direct analysis of certain patient samples like blood.

3.2.2. Amplification NATs—

In contrast to the hybridization-based techniques, many other NATs rely on DNA amplification to generate a readout, commonly through gel electrophoresis or an increasing fluorescent signal. While some tests target rRNA (directly as complementary DNA (cDNA) or as the gene) like with the PNA-FISH based tests, amplification allows lower abundance, species-specific genes to be used.[53] This opens the possibility of determining the antibiotic susceptibility of pathogens through the detection of common resistance-conferring genes.[208] The earliest amplification based tests were introduced in the mid-1990s for the specific detection of *M. tuberculosis*,[209] and a number of tests capable of identifying a wide range of pathogens and resistance markers have since been released.[53,57,208,209] The advent of real-time PCR allowed for further improvements to amplification based technologies, eliminating the need for post-amplification analysis through techniques like gel electrophoresis and allowing for quantitative results.[210] An early example of a commercial real-time PCR device is the Cepheid Xpert MRSA/SA test, which can identify the presence *S. aureus* and the *mecA* gene for methicillin resistance in an hour from a positive blood culture.[211] Real-time PCR systems with multiplexing to allow the detection of multiple pathogens have also been developed, such as the SeptiFast system for the identification of the 25 most common pathogens responsible for blood stream infections.[212]

There are several important considerations when discussing amplification based techniques, which have been discussed in several comprehensive reviews.[53,208,213] One issue is the amount of sample handling required to prepare DNA and set up reactions. The possibility of contamination is relatively high during this time so significant effort has gone into the development of automated, cartridge based systems for DNA extraction and preparation.[213] Generally, free DNA is not removed prior to lysis of bacterial cells so many amplification-based tests will report on the presence of dead cells and DNA from recent infections.[208] This can be advantageous in some cases, especially if a patient sample is collected after antibiotic treatment has already begun, but can also lead to false positives as the presence of small

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amounts of bacterial DNA in samples like blood is not always indicative of infection.[214] Another consideration concerning resistance profiling is that only known resistance genes can be targeted, so the reliability of susceptibility determination through these methods may be questionable for some pathogens. Financially, real-time PCR instruments require a moderate initial investment and highly multiplexed tests for the detection of numerous pathogens can carry a significant cost per sample. Finally, as with direct hybridization techniques, only specifically targeted pathogens can be detected.

A method to circumvent the need to target specific primers to each species is to use broad range PCR. This approach relies on amplification of common genes such as the 16S RNA with primers that bind in conserved regions, similar to 16S typing for bacterial identification. Rapid species determination can then be performed with high-resolution melt analysis (HRMA) by comparing the melting curves of a set of amplification products to an existing database.[215,216] Sequence differences between species lead to slight changes in the melting temperature for each amplification product and have been used to differentiate clinically relevant pathogens.[189] The accuracy of the technique is inherently limited by the quality of the reference database and a large number of known clinical isolates will need to analyzed and recorded to ensure sufficient species coverage for comparison. Strain variants with polymorphisms in the amplified gene regions could pose a problem however, and polymicrobial samples are impossible to analyze with HRMA, severely limiting its applicability.[217] Other detection methods for broad range PCR exist, including sequencing and electrospray ionization (ESI)-MS (discussed in section 3.3.2).

Moving a step beyond sequencing with broad range PCR, whole genome sequencing (WGS) with next-generation sequencing (NGS) methods has the potential to supplant traditional clinical microbiology as the gold standard in identifying the causative agent(s) of infectious disease.[55,218] After sequencing, the infective species can be identified with the highest degree of confidence by alignment to a reference genome (if available) or by multilocus sequence typing (MLST) if a member of the same species has not yet been sequenced.[55,219] In addition to species identification, WGS provides information on the presence of resistance markers and virulence factors and is an unparalleled tool for differentiating strains for epidemiological purposes.[55,220] A number of platforms for NGS have been developed utilizing different chemistry, including 454, Illumina, Ion Torrent, and PacBio, among others.[221,222] Currently, WGS is too slow and expensive for routine pathogen identification, but may be a viable option in the near future if the rapid advancement in sequencing technology over the past decades continues.[55] Pathogen identification directly from patient samples using a metagenomics approach removes the >24 h delay required for DNA extraction from culture,[223,224] while improvements in NGS have already drastically reduced the time and price required for sequencing (a single bacterial genome with sufficient depth coverage can now be obtained in less than a day for $200 by academic core facilities, with cost-savings truly becoming significant when many bacteria are sequenced).[55,218,225] With continued advances in NGS and automated data analysis software,[223] it appears that WGS is posed to replace all other NAT diagnostics.

While amplification techniques have proven to be accurate enough to be used with narrow-spectrum antibiotics, the time currently required for amplification is of concern. Many

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commercial tests are designed to work with the high bacterial counts available after initial culture and can give a result in as little as an hour,[211,226] but the delay from culture time is significant. Testing directly from patient samples suffers from low starting bacterial counts and the presence of substances inhibitory to amplification, such as heme in blood.[210] The presence of vast excesses of host DNA can also be problematic in PCR assays.[210,227] Several tests have been developed that overcome these issues and can be used directly on blood samples but they require much longer amplification times of 6–12 h.[53] This is still an improvement over waiting for cultures to grow however and can likely be further improved through methods of isolating bacteria or high quality bacterial DNA from patient samples, as discussed in section 3.2.3. No resistance determination is available in the tests currently marketed for use on blood but could presumably be included for known resistance genes in the future.

3.2.3. Bacterial isolation from patient samples—While some clinical sample types such as urine from UTIs can contain high bacterial counts (>10^5 colony forming units (CFU)/mL),[228] many samples have far fewer bacteria present. Blood in particular can have as little as 1–10 CFU/mL of an invading pathogen in adult sepsis patients.[229] Therefore, methods for isolating high quality bacterial DNA from samples that often contain large amounts of human DNA can improve the reliability of NAT methods. The simplest way to achieve this is through the coupling of standard DNA purification systems with the selective removal of human DNA. One approach is to gently lyse human cells while leaving bacterial cells intact, followed by enzymatic or chemical degradation of human DNA. After washing, bacterial cells are lysed through a more vigorous method and the DNA is isolated.[230,231] Another approach involves exploiting the differences in methylation of cytidylate-phosphate-deoxyguanylate (CpG) motifs by human CpG-binding protein. Unmethylated prokaryotic CpG motifs are selectively bound with an immobilized protein, allowing human DNA to be washed away to give an affinity purification of the bacterial DNA.[230,232]

An alternative strategy for the isolation of bacteria or bacterial DNA from patient samples involves the use of microfluidics technologies. Patient samples can be passed through devices that contain bacterial capture materials, including antibodies,[233–235] synthetic ligands,[236] or even human opsonin.[237] After capture, human cells and other sample components can be readily washed away before release of bacterial DNA via cell lysis.[234] A different microfluidics based approach that does not rely on specific bacterial capture but rather on the intrinsic physical properties of the bacterial cells was recently reported.[203] The method utilizes inertial lift forces to separate bacteria from blood cells based on size differences. This type of method also has the advantage of collecting intact cells, which could then potentially be used in non-DNA based detection methods such as MALDI.

3.3. Mass spectrometry-based technologies

The advent of so called “soft” ionization methods that are capable of ionizing large biomolecules without significant fragmentation has made the development of MS-based methods appealing for pathogen identification. These methods are based on the detection of sets of genus- or species-specific biomolecules, typically proteins or nucleic acids.[190,238] The collected spectra are compared to a database assembled from known isolates to identify

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the species present, similar to PCR HRMA. Although MS methods require a large initial investment with the purchase of instrumentation, they have the advantage of extremely low per-sample costs and very fast turnaround times. In contrast to NATs, MS-based methods do not require specific probes for each species of interest and can thus theoretically identify any species in a sample without any prior knowledge if robust databases are available. Two different general approaches to MS-based identification have been developed thus far, matrix-assisted laser desorption/ionization (MALDI)-MS and PCR ESI-MS, although MALDI-based techniques have been more extensively investigated.

3.3.1. MALDI-MS—MALDI-MS functions through desorption and ionization of sample molecules after laser irradiation with the assistance of matrix compounds followed by analyte detection, typically in a time of flight (TOF) analyzer. The application of MALDI-TOF-MS to clinical microbiology has been extensively reviewed.[190,239,240] Differences in bacterial proteomes are commonly used for species identification with MALDI-TOF-MS, although other biomolecules can also be detected. Generally, the focus is on ribosomal proteins as they are abundantly expressed throughout all stages of growth.[190] Since no prior knowledge of the organism in a sample is needed, fungi can also be identified without additional testing components assuming a reference database including the organisms of interest is available.[239] While samples can be analyzed directly using whole cells, a protein extraction step improves signal intensity and reproducibility and is required for some organisms.[239] Sample preparation and analysis with MALDI-TOF-MS is extremely rapid, generally taking less than 15 minutes. With a sufficiently extensive database, the presence of any pathogen in a sample can be accurately detected.[241] Several MALDI-TOF-MS instruments designed for microorganism detection are available, including the Bruker BioTyper and the Shimadzu Axima Assurance system.[242]

However, there are several important limitations in the application of MALDI-TOF-MS for diagnostics that still need to be overcome. Currently, MALDI-TOF-MS for bacterial identification generally requires a high CFU count and thus is often not feasible directly on patient samples.[190,240] Samples are usually analyzed after culture, which adds a significant amount of time to obtain results. However, several studies have reported success when analyzing urine[243–246] and cerebral spinal fluid[247] samples directly. Bacteria could potentially be concentrated from patient samples as well using microfluidics technologies as described in section 3.2.3 to help circumvent the lack of sensitivity. Another issue facing the widespread implementation of MALDI-TOF-MS as a clinical tool is the often poor reproducibility between labs, as different sample preparation techniques and instrument types can cause substantial variation in analyte detection,[248,249] although standardization between labs (perhaps through the Clinical and Laboratory Standards Institute or a similar organization) will improve this. The detection of antibiotic resistance is difficult with MALDI-TOF-MS but strategies to accomplish this have been advanced,[250] including the detection of methylation on rRNA[251] and reaction monitoring of β-lactam hydrolysis.[252] Differentiation between highly similar species and deconvolution of polymicrobial samples also pose significant challenges. Despite these problems, MALDI-TOF-MS has been successfully validated as a highly accurate method for pathogen identification in numerous clinical samples and several commercial platforms are currently available.[190,241,242]

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MALDI-TOF-MS systems have been adopted in many advanced clinical labs and reductions in size and cost of the instruments will enable smaller labs to utilize this technology as well. If improvements in analyzing patient samples directly can be made, the ability to identify any pathogen in the available databases makes MALDI-TOF-MS a promising possibility for diagnosis prior to narrow-spectrum antibiotic use.

3.3.2. PCR ESI-MS—ESI is another MS ionization method whereby a high voltage is used to disperse a sample into an aerosol containing charged droplets.[253] It has been used as a general detection method for bacterial identification after PCR amplification with broad range primers, similar to PCR HRMA.[217] The amplified DNA is analyzed by ESI-MS and species identification is achieved by comparing the experimental mass to charge (*m/z*) ratio of the PCR products to the expected *m/z* ratio based on the gene sequence.[208,254,255] Like with MALDI-MS based methods, a robust database is required, but databases for ESI-MS can be built from existing sequencing information in addition to clinical isolates.[208] This can ease the identification of strain variants and results that do not have an exact match in the database can have the nearest neighbor identified by a BLAST like search algorithm.[208] As with PCR HRMA and MALDI-MS, no prior knowledge of the sample species is required before testing.

However, the requirement of an amplification step introduces many of the issues described in section 3.2.2 for amplification based NAT techniques, such as sample processing and the time needed for amplification. The ESI-MS step itself it very rapid however and can be set up to run in an automated fashion. Several sets of primers for different gene regions are used to mitigate the risk posed by genotypic strain variants, although it is still possible that a divergent strain could be misidentified.[255,256] Resistance determination can be performed in a manner similar to other amplification based methods by including primers for specific resistance conferring genes at the cost of increased complexity and number of reactions that must be run on each sample.[256] PCR ESI-MS also has the advantage of working well with mixtures of organisms, with the identification of both species and the relative ratios of abundance usually determined.[256,257] As with MALDI-MS instruments, ESI-MS instruments tend to be large and expensive, but improvements in miniaturized ESI-MS units[258] and in amplification technologies offer the potential for PCR ESI-MS to be a rapid point-of-care diagnostic. The Iridica system from Abbott Molecular is currently on the market and is specifically designed for pathogen identification directly from patient samples although it is not yet available in the United States.[259]

3.4. Alternative detection and identification technologies

Outside of NAT and MS-based methods, there are a number of other promising technologies that could be utilized for bacterial detection and identification prior to narrow-spectrum antibiotic use. One established method for diagnosis is rapid antigen testing, which relies on a visible readout for antibody-antigen binding. Rapid antigen testing has been used for years for *S. pyogenes* and provides results in ~15 min at the point-of-care, compared to 1–2 d for traditional culture on blood agar plates.[260–262] However, antigen testing in its current form only exists for a few select pathogens, likely due in part to the limited scope of such tests. It is thus generally used where clinical signs point to a certain type of infection, as with

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pharyngitis. Rapid antigen testing could be extended to additional pathogens (e.g. *E. coli* for UTIs) or potentially even multiplexed to allow broader identification.

The remaining techniques discussed in this section are relatively new and have not undergone the extensive testing with patient samples that most of the previous methods have, but they offer some promising possibilities for future development. Microring resonators were discussed in section 3.2.1 as an emerging technology for bacterial identification by hybridization of RNA but they could also be used for the direct detection of pathogens with species-specific antibody functionalized rings. This concept has been demonstrated for detection of intact bacterial cells[205] and virus particles.[263] Microring resonators designed around this concept rather than RNA detection would eliminate the need for an RNA isolation step and could be extremely rapid, but would require highly specific antibody generation for each pathogen of interest.

Another method that is being applied to pathogen detection and identification is T2 magnetic resonance (T2MR)-based biosensing. T2MR measures the transverse relaxation time of the nuclear magnetic resonance signal of water and is sensitive to changes in the microenvironments in aqueous solutions.[264] Recently, DNA functionalized nanoparticles that cluster upon binding of target nucleic acids were reported, resulting in easily detected T2 relaxation rate changes.[265] In combination with a specially designed, portable T2MR instrument that is compatible with standard PCR tubes, detection of *Candida* species at concentrations <5 CFU/mL in blood directly from patient samples was reported.[265,266] Although an amplification step is required, results could be obtained in <3 h, which is less than half the time required for real-time PCR based methods. The method is also currently in development for bacterial detection by T2 Biosystems. Although the ability of T2MR to determine resistance profiles has not been discussed in the literature, it would theoretically be possible by functionalizing additional nanoparticles with DNA oligomers targeting known resistance genes.

The final technology we will discuss is integrated comprehensive droplet digital detection (IC 3D).[267] IC 3D provides a readout for highly sensitive bacterial detection through single fluorescent particles suspended in picoliter droplets. The fluorescent droplets are produced by mixing a DNAzyme sensor solution and the sample solution with lysis buffer in a microfluidic channel that encapsulates them into the droplets. The DNAzymes are activated by specific target bacterial molecules, which results in the cleavage of a nucleic acid substrate to release a fluorophore from its associated quencher. The fluorophore is then detected by a recently developed 3D particle counter.[268] The entire process takes around 4 h for bacterial identification and is sensitive enough to detect <10 CFU/mL while also providing quantitative results directly from blood samples. While this method has many promising advantages, it will need to be developed into a multiplexed system for multiple species detection and suffers from the requirement for a specific DNAzyme for each species.

4. Conclusions

Due to the rapid evolution of resistance to antibiotics, we continually need new approaches to fight bacterial infections. Given the difficulty in developing new antibiotics, innovative

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methods to help design/discover/develop new drugs that slow resistance acquisition will always be welcome. Narrower spectrum drugs show promise, but will require improved diagnostics to help rapidly and accurately identify pathogens prior to treatment. Since narrow-spectrum antibiotic development and pathogen detection are becoming increasingly intertwined, both will benefit by advances in the other. However, focusing solely on narrowing the spectrum of antibiotics is not sufficient; the highest levels of efficacy and safety required for current antibiotics must also be maintained. New diagnostics need to demonstrate superb accuracy and sensitivity; otherwise results will likely be ignored without additional, supporting tests to confirm the diagnosis. There is currently a national spotlight on these issues due to recent executive and congressional actions, hopefully spurring great progress in the near future.

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Abbreviations

Cas	CRISPR-associated protein
cDNA	complementary DNA
CFU	colony forming units
CpG	cytidylate-phosphate-deoxyguanylate
CRISPR	clustered, regularly interspaced, short palindromic repeats
ESI	electrospray ionization
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species
FDA	Food and Drug Administration
FISH	fluorescence in situ hybridization
GAIN	Generating Antibiotic Incentives Now
HGT	horizontal gene transfer
HRMA	high-resolution melt analysis
IC 3D	integrated comprehensive droplet digital detection
MALDI	matrix-assisted laser desorption/ionization
MDR	multiple drug-resistance
MLST	multilocus sequence typing
MRSA	methicillin-resistant *S. aureus*

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MS	mass spectrometry
NATs	nucleic acid-based technologies
NGS	next-generation sequencing
PCR	polymerase chain reaction
PNA	peptide nucleic acid
rRNA	ribosomal ribonucleic acid
T2MR	T2 magnetic resonance
TB	tuberculosis
UTI	urinary tract infection
WGS	whole genome sequencing

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Biographies

Tucker Maxson was born in Oakland, California, USA in 1988. He obtained his B.S. in Chemistry from the University of California, San Diego in 2010, where he performed research under Prof. Yitzhak Tor. He is currently pursuing a Ph.D. in chemical biology in the research group of Prof. Douglas Mitchell at the University of Illinois at Urbana-Champaign. His research interests include natural product discovery and the development of novel methods for fighting bacterial infections.

Douglas Mitchell was born near Pittsburgh, Pennsylvania in 1980. He received his B.S. in Chemistry from Carnegie Mellon University in 2002. After a short internship in medicinal chemistry at Merck Research Laboratories, he obtained his Ph.D. from the University of California, Berkeley in 2006 while working with Prof. Michael Marletta. For postdoctoral studies, he worked with Prof. Jack Dixon at the University of California, San Diego. Prof. Mitchell joined the Department of Chemistry faculty at the University of Illinois at Urbana-Champaign in 2009 and was promoted to Associate Professor in 2015. He holds an affiliate position in the Department of Microbiology and is a faculty member of the Carle R. Woese Institute for Genomic Biology. The primary objective of the Mitchell lab is to use a blend of chemical and biological approaches to address the alarming rise in antibiotic resistance. Primarily through genomics-based approaches, the Mitchell lab seeks to structurally and functionally characterize novel antibiotic natural products. The lab is also interested in biosynthetic mechanistic enzymology, with the goal of engineering new chemical and biological functionaility to existing natural product scaffolds.

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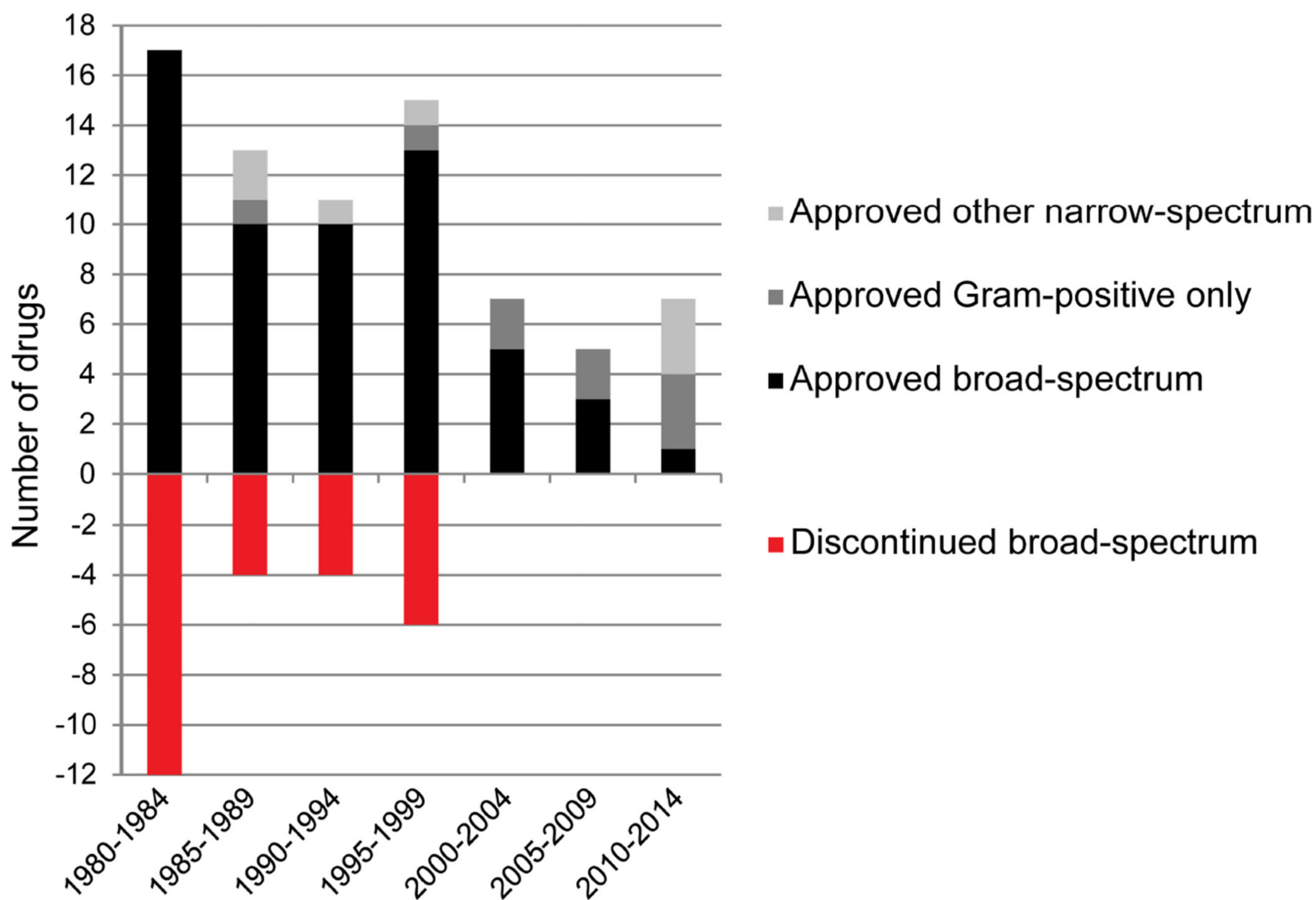

Fig. 1.
US FDA approval and discontinuation of antibiotics from 1980 to 2014. Approvals are shown in black. Discontinued antibiotics are shown in red as negative values.[27] Approvals are split by clinically useful activity into broad-spectrum, narrow-spectrum that target Gram-positive species only, and all other narrow-spectrum drugs (includes antimycobacterials). No narrow-spectrum antibiotics were discontinued during this time period.

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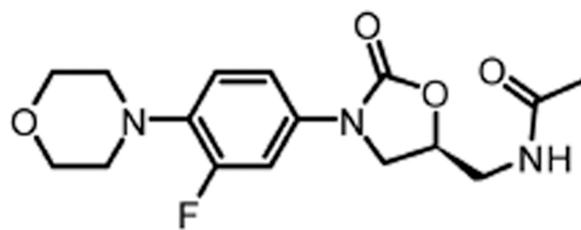

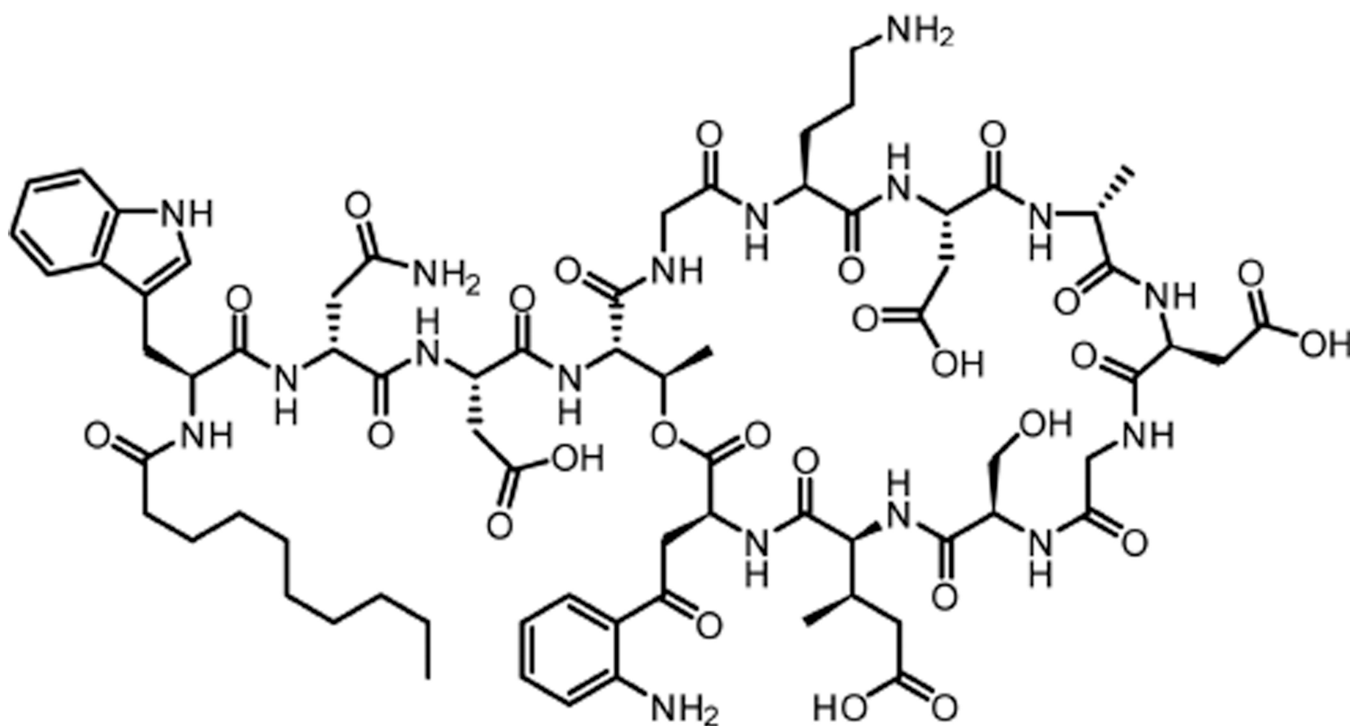

Fig. 2.
Structures of linezolid and daptomycin.

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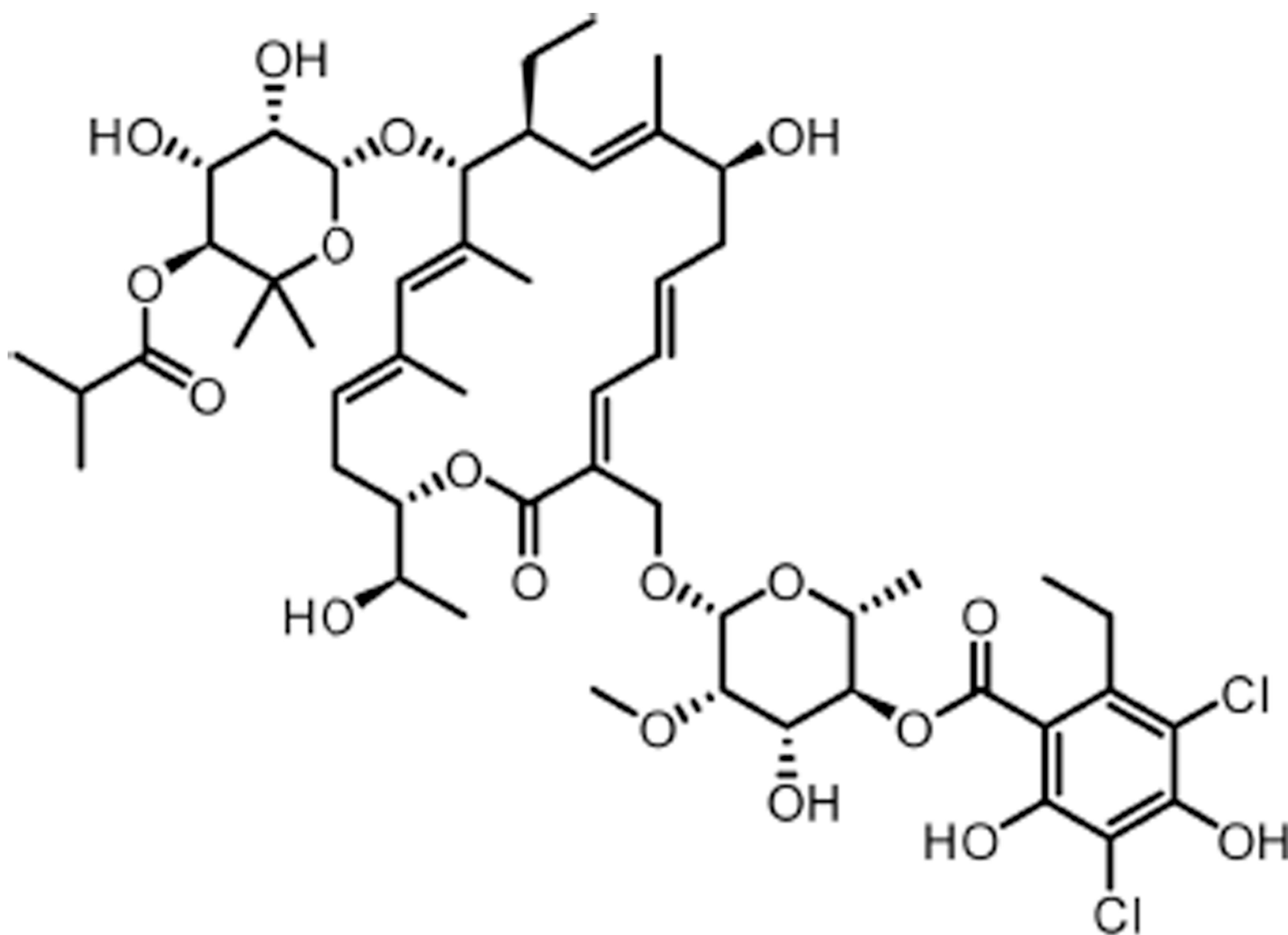

Fig. 3.
Structure of fidaxomicin.

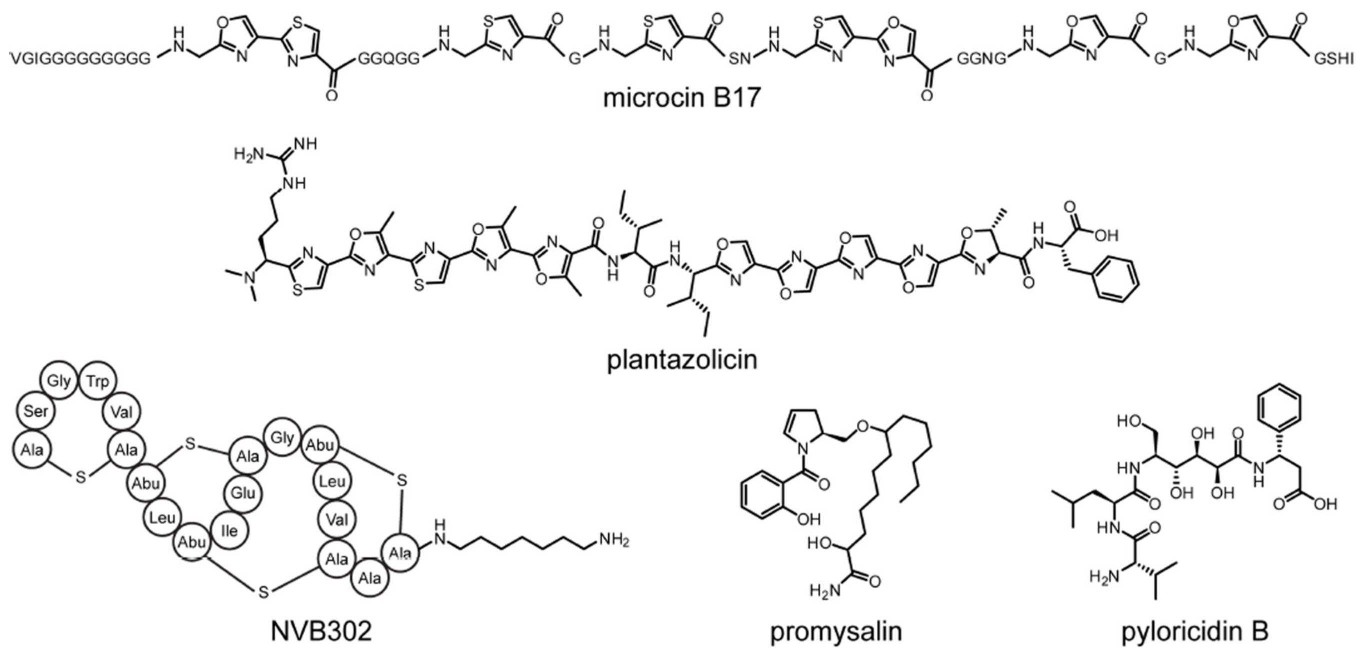

Fig. 4.
Structures of pathogen-specific antibiotics.

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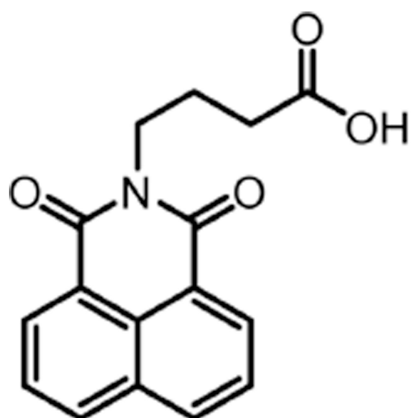

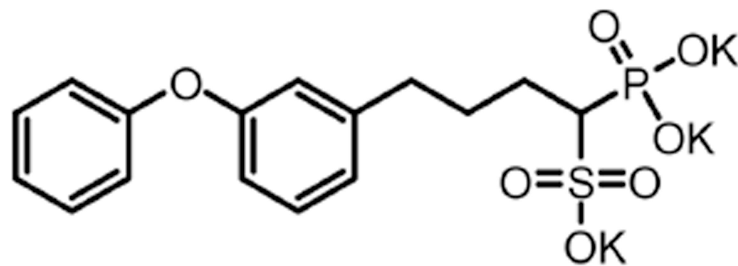

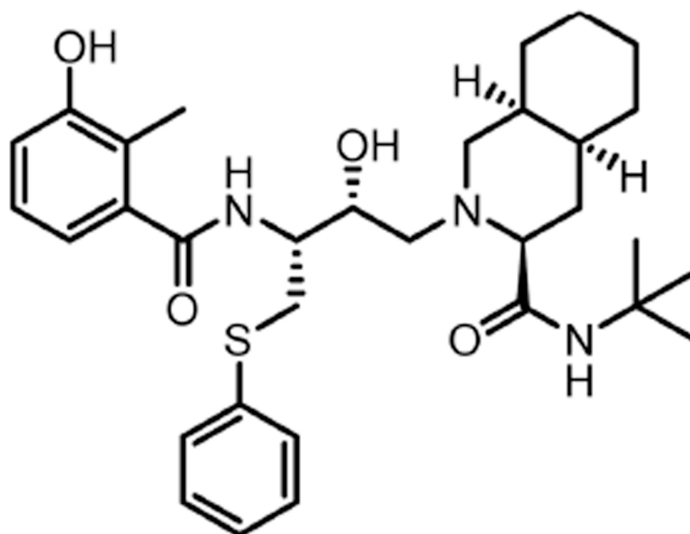

Fig. 5.
Structures of anti-virulence agents.

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Table 1

Summary of molecular diagnostic techniques.

Technique	Turnaround time	Number of species possible per assay	Resistance detection	Approximate Instrumentation cost
PNA-FISH	< 1 h after blood culture	1	No	< $1,000
NanoString	8 h	Undeveloped, > 800 targets possible	Yes	> $100,000
Microring resonators (RNA hybridization)	< 1 h	Undeveloped, multiplexing possible	Yes	~ $100,000
PCR from culture	1 – 3 h	> 50 for commercial assay	Yes	$20,000 – 50,000 (for real time)
PCR from blood	6 – 12 h	> 300 for commercial assay	Currently no	$20,000 – 50,000
PCR HRMA	3 h after culture	All	No	$20,000 – 50,000
WGS	> 24 h	All	Yes	> $100,000
MALDI-MS	< 15 min after culture	All	In development	> $100,000
PCR ESI-MS	5 h after culture	All	Yes	> $100,000
Rapid antigen testing	< 30 min	1	No	N/A
Microring resonators (Direct pathogen detection)	< 1 h	Undeveloped, multiplexing possible	No	~ $100,000
T2MR	3 h	Undeveloped, multiplexing possible	Yes	Undeveloped
IC 3D	4 h	Undeveloped, multiplexing possible	No	Undeveloped