The Challenge of Emerging Infections and Progressive Antibiotic Resistance

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"If we do not change our direction, we are likely to end up where we are headed"

ancient Chinese proverb

Introduction

Our collective vulnerability to the threat of emerging microbial pathogens remains disturbingly evident as we enter the twenty-first century. Despite two centuries of knowledge about the germ theory of disease, breaking the genetic code, and sequencing the genomes of virtually every major bacterial and viral pathogen capable of causing disease in humankind, we still find ourselves susceptible to infectious diseases. Densely concentrated cities with interconnected human societies linked by international aviation put us at continued risk from future epidemics that will inevitably occur [1]. The ever expanding population growth of our species will force environmental change as we venture into sparsely inhabited rainforests, populate remote ecosystems and cultivate natural habitats to support our voracious human appetite for goods and services. Global warming, environmental degradation and land development along with human upheavals and natural calamities will create new outbreaks with novel pathogens and renew the spread of ancient scourges like cholera [2] and plague [3].

Numerous examples of intercontinental spread of microbial pathogens within the last five years alone give notice of the susceptibility of human populations to emerging infectious diseases (Table 1) [4–20]. This is perhaps best exemplified by the tragic events set into motion in late 2002 when a previously unidentified, obscure, animal coronavirus (now known as severe acute respiratory syndrome [SARS]-CoV) was first introduced into an unsuspecting human population in Southern China [9]. Current molecular evidence indicates that a food handler in an exotic food 'wet market' in Guangdong Province probably first became infected by an animal coronavirus from a civet cat. This newly derived animal virus was adapt at infecting humans and was efficiently spread person-to-person by infected aerosol [10]. An ill Chinese physician from the affected region traveled to Hong Kong to attend a wedding. While spending a single night in Amoy Garden Hotel in the city, this infected individual appeared to spread the virus to at least 12 other hotel guests. Over the next several days these people returned to their homes in five different countries incubating the SARS-CoV

pathogen in their respiratory secretions. Over the next 3–4 months, this newly acquired coronavirus spread to over 27 countries worldwide and caused over 8000 cases of SARS resulting in nearly 800 deaths in early 2003 [9]. Through a global effort from a large number of very diligent public health officials and laboratory scientists, the outbreak ended within a year and has yet to be seen again, except for occasional laboratory-acquired accidents [9].

A diverse array of pathogens has produced recent outbreaks and concerns for our vulnerability to pathogens within the global village we occupy and share with other flora, fauna and microorganisms (Table 1). The spread of mosquito-

Table 1. Emerging infectious disease threats in the 21st century

Disease	Causative Organism	Cause and Outcome
Avian influenza [4-7]	Influenza A (H_5N_1)	Risk of pandemic influenza; sporadic human cases of avian flu in Asia -mortality rates>70%
Severe acute respiratory syndrome (SARS) [8–10]	SARS associated coronavirus	Risk of spread of zoonotic viruses; outbreak from a southern China to world-wide epidemic in 2003 – 8000 cases and 800 deaths
Monkey pox [11]	Orthopox virus	Risk of exotic pet trade; outbreak in wild rodents and humans in Mid-Western USA from sale of Gambian Giant rats from Africa
West Nile Virus (WNV) [12, 13]	Mosquito-borne flavivirus	Risk of international spread; WNV from Africa to New York in 1999, thousands of cases and hundreds of deaths in North America over next 5 years
Inhalational anthrax [14, 15]	Intentional release of *Bacillus anthracis* spores in USA mail system	Vulnerability to bioterrorism; 11 cases, 5 fatalities in Oct-Nov 2001, perpetrator never identified
Hemorrhagic fever outbreaks [15, 16]	Ebola virus – Zaire (filovirus)	Disruption of ecosystems; repeated outbreaks in Gabon and Congo rainforests in 2001–2004
Antibiotic resistant bacteria, viruses, fungi [17–20]	Vancomycin-resistant *S. aureus* Oseltamivir-resistant influenza Azole-resistant *Candida spp.*	Misuse of antimicrobials promote spread of resistance genes – community outbreaks now occur

borne West Nile virus in North America [12, 13], prion-related food-borne variant Creutzfeldt-Jakob Disease [21], and hemorrhagic fever viruses [16] are a constant reminder of our susceptibility to pathogens that naturally reside in other animal species. The omnipresent fear of the next pandemic of influenza has been heightened by recent evolutionary changes in virulence and transmissibility of avian flu viruses [22].

Standard chemotherapeutic regimens for infectious diseases may not reliably rescue persons with severe infections in the new millennium. Community and nosocomial outbreaks of multidrug resistant pathogens as evidenced by methicillin and vancomycin resistance [17] in *Staphylococcus aureus* and resistance to the new anti-viral neuraminidase inhibitors [18, 19] by recent influenza isolates are cause for real concern. The care of hospitalized, critically ill patients is likely to fundamentally change if current trends in the progressive emergence of antimicrobial resistance to commonly prescribed antibiotics are not significantly altered in the near future. Regrettably, there is little evidence that the situation is likely to change unless concerted efforts are taken on several fronts to reverse the current trajectory of increasing antibiotic resistance [17, 20].

The Genetics of Antibiotic Resistance

The fitness of a microorganism is dependent upon its capacity to genetically adapt to rapidly changing environmental conditions. Antimicrobial agents exert strong selective pressures on microbial populations, favoring those organisms that are capable of resisting them. Genetic variability may occur by a variety of mechanisms. Point mutations may occur in a nucleotide base pair, which is referred to as *micro-evolutionary change* [23]. These mutations may alter the target site of an antimicrobial agent, altering with its inhibitory capacity.

Point mutations inside or adjacent to the active sites of existing beta-lactamase genes (e.g., genes for TEM-1, SHV-1) have generated a remarkable array of newly recognized extended-spectrum beta-lactamases [23]. Beta-lactam antibiotics have been known for almost 80 years and their widespread use has created selection pressures on bacterial pathogens to resist their inhibitory actions. At least 267 different bacterial enzymes have now been characterized that hydrolyze beta-lactam antibiotics [24]. The hydrolyzing enzymes exist in four basic molecular classes and are classified as listed in Table 2. The enzymes are either serine hydrolases (class A, C, and D) or zinc containing metalloenzymes with a zinc-binding thiol group its active site (class B enzymes). The microevolutionary events that account for the differential activities of this array of beta-lactamases have been carefully studied, and these bacterial enzymes now even have their own internet website devoted specifically to their molecular properties (http://www.lahey.org/studies/webt.htm).

Beta-lactamase activity has become so ubiquitous among bacterial populations that it has prompted the development of specific beta-lactamase inhibitor compounds (clavulanate, tazobactam and sulbactam) in an effort to combat this common bacterial resistance mechanism. This has been countered by the generation of inhibitors of these beta-lactamase inhibitors by multidrug-resistant

Table 2. The functional classification scheme for beta-lactamases

Group	Enzyme Type	Clavulanate inhibition	Molecular Class	Common Examples
1	Cephalosporinase	No	C	*Enterobacter cloacae* P99
2a	Penicillinase	Yes	A	*Staphylococcus aureus*
2b	Broad-spectrum	Yes	A	SHV-1, TEM-1
2be	Extended-spectrum	Yes	A	*Klebsiella oxytoca* K1
2br	Inhibitor-resistant	Diminished	A	TEM-30 (IRT-2)
2c	Carbenicillinase	Yes	A	AER-1, PSE-1, CARB-3
2d	Cloxacillinase	Yes	A or D	OXA-1
2e	Cephalosporinase	Yes	A	*Proteus vulgaris*
2f	Carbapenemase	Yes	A	IMI-1, NMC-A, Sme-1
3	Carbapenemase metalloenzymes	No	B	*Stenotrophomonas maltophilia* L1, IMP-1
4	Penicillinase	No		*Burkholderia cepacia* , SAR-2

(see references [23, 24])

bacteria [23] in the ongoing conflict between pathogens and chemotherapeutic strategies to eradicate these microorganisms.

Recently it has been demonstrated that at least some bacterial populations have the capacity to increase their mutation rates during times of environmental stress such as exposure to an antibiotic. This stress response is known as the 'SOS' response or transient hypermutation [25]. It is highly advantageous for the organism to increase the rate of genetic variation at times of unfavorable environmental conditions. It is possible for bacteria to upregulate the pace of evolution in an attempt to develop a clone that can resist the action of an antibiotic. The DNA polymerase in such organisms has reduced fidelity of replication and subsequently an increased rate in the mutational occurrences as a result of excess nucleotide mispairing. The recombination system of bacteria (the *recA* system) becomes less restrictive in the degree homology between DNA sequences before a crossover event is permitted to occur. A flurry of mutational events occur in stressed bacteria in a final attempt to generate a resistant subpopulation of bacteria in the presence of an environmental challenge such as the presence of a new antibiotic. This process has even been phenotypically linked with alterations in growth rate and biofilm formation in some strains of *Pseudomonas aeruginosa* [26].

A second level of genomic variability in bacteria is referred to as a *macro-evolutionary* change and results in whole-scale rearrangements of large segments of DNA as a single event. Such rearrangements may include inversions, duplica-

tions, insertions, deletions, or transposition of large sequences of DNA from one location of a bacterial chromosome or plasmid to another. These whole-scale rearrangements of large segments of the bacterial genome are frequently created by specialized genetic elements known as *transposons* or *insertion sequences*, which have the capacity to move independently as a unit from the rest of the bacterial genome [23].

Acquisition of foreign DNA sequences from the extracellular environment may be taken up by naturally competent bacteria (e.g., some streptococci and neisserial organisms) by transformation. These sequences can then become integrated into the host genome into homologous sequences by the generalized recombination and DNA repair system bacteria. Inheritance of these foreign DNA elements further contributes to the organism's ability to cope with selection pressures imposed upon them by antimicrobial agents [23].

A third level of genetic variability in bacteria is created by the acquisition of foreign DNA carried by plasmids and bacteriophages. These extrachromosomal DNA elements provide ready access to disposable yet potentially highly advantageous genes including antibiotic resistance genes from plasmids or phage particles. These elements are autonomously self-replicating, and they can remain unattached in the cytoplasm of bacterial cells or integrate directly into the chromosome of the bacterial host. They have the capacity to replicate and move independently from the chromosome adding further variability to the entire bacterial genomic DNA. Evidence from whole genome sequencing projects indicates that these genomic rearrangements, bacteriophage sequences and insertion sequences are commonplace in bacterial chromosomes [27].

These genetic variations provide bacteria with the seemingly limitless system to alter their genomes, rapidly evolve and develop resistance to virtually any antimicrobial agent. Recent examples of vancomycin-resistance in enterococci [23], *S. aureus* [27], and extended spectrum beta-lactamases [23], carbapenemase production [28] and transferable quinolone resistance in *P. aeruginosa* and enterobacteria [23] attest to the capacity of microorganisms to adapt to environmental stresses induced by antibiotic exposure. Viruses [19] and fungi [20] are also quite capable of rapid antimicrobial resistance development and these resistance capacities pose additional threats in the management of ICU patients with serious infections from a variety of potential pathogens [29].

The Origins of Antibiotic Resistance Genes and Mechanisms of Resistance

Antibiotic resistance genes probably arose from detoxifying enzymes or synthetic enzymes with altered substrate specificity by critical mutations or recombination events resulting in the formation of mosaic genes with entirely new functions [30]. Altered penicillin binding proteins that mediate beta-lactam resistance in multiple bacterial genera (e.g., methicillin-resistant *S. aureus* [MRSA], penicillin-resistant streptococci and pneumococci, chromosomal resistance in gonococci) may have evolved from gene fusions for penicillin binding proteins involved in bacterial cell wall synthesis [23]. Another common resistance strategy is a change in the regulation of metabolic activity of an enzyme system that

is affected by the antibiotic. Increasing the rate of folate precursor synthesis, for example, can overcome the inhibitor effects of sulfa drugs and trimethoprim [30].

Many common antibiotic resistance genes were accidentally acquired ('stolen') from antibiotic producing bacteria. Streptomyces and related soil bacteria are the source of many standard antimicrobial agents in use in clinical medicine today. These bacteria have co-evolved the capacity to synthesize antibiotics along with the necessary resistance genes to protect their own metabolic machinery from the very antibiotic they produce. The resistance genes from these antibiotic producing bacteria provide a ready genetic blueprint to resist the target antibiotic if susceptible bacteria can acquire these resistance genes. Recent evidence confirming that this does indeed occur was found by Yokoyama and colleagues in Japan during an investigation of a sudden outbreak of *P. aeruginosa* with high-level resistance to essentially all the clinically available aminoglycosides [31]. These investigators discovered that the resistant strain had acquired a new methylase gene that blocked the binding site for inhibition by aminoglycosides on a specific sequence on 16S ribosomal RNA. This identical mechanism and highly homologous gene is found in aminoglycoside-producing strains of Streptomyces and related bacteria.

Detoxifying Enzymes

At least seven distinctive mechanisms of antibiotic resistance have been described in bacteria and are summarized on Table 3. Detoxifying enzymes are used to degrade beta-lactams [24], and modify aminoglycosides so they no longer enter bacterial membranes and attach to their ribosomal target. There are over 30 such enzymes identified that can inhibit aminoglycosides by one of three general reactions: *N*-acetylation, *O*-nucleotidylation, and *O*-phosphorylation [23]. Detoxifying enzymes are also one of the resistance mechanisms against chloramphenicol, and are rarely utilized by certain bacterial strains to inactivate macrolides, lincosamides, tetracyclines and streptogramins.

Decreased Permeability

It was recognized early in the history of antibiotic development that penicillin is effective against Gram-positive bacteria but not against Gram-negative bacteria [23]. This difference in susceptibility to penicillin is due in large part to the outer membrane, a lipid bilayer that acts as a barrier to the penetration of antibiotics into the cell. Situated outside the peptidoglycan cell wall of Gram-negative bacteria, this outer membrane is absent in Gram-positive bacteria. The outer portion of this lipid bilayer is composed principally of lipopolysaccharide (LPS) made up of tightly bound hydrocarbon molecules that impede the entry of hydrophobic antibiotics, such as penicillins or macrolides.

The passage of hydrophilic antibiotics through this outer membrane is facilitated by the presence of porins, proteins that are arranged so as to form water-

Table 3. Mechanisms of antibacterial resistance by major drug class

	β–lactam	Amino-glycoside	Sulfa/TMP	Quino-lone	Macro-lide	Glyco-peptide	TCN
Enzymatic inactivation	+++	+++	–	–	+ (Gram-neg)	–	+
Im-permeable	+ (Gram-neg)	+ (Gram-neg)	+ (Gram-neg)	+ (Gram-neg)	++ (Gram-neg)	++ (Gram-neg)	+
Efflux	+	+	–	+	++	–	+++
Altered target site	++	++	+++	+++	+++	+++	+
Protected target site	–	–	–	+	–	–	++
Excess target	–	–	++	–	–	+	–
Bypass process	–	–	+	–	–	–	–

Gram-neg: Gram-negative bacteria; TMP: trimethoprim; TCN: tetracycline; +++: most common mechanism; ++: common; +: less common, –: not reported (see reference [23])

filled diffusion channels through which antibiotics may traverse [23]. Bacteria usually produce a large number of porins with differing physiochemical properties, permeability characteristics and size; approximately 10^5 porin molecules/cell for *Escherichia coli*. Bacteria are able to regulate the relative number of different porins in response to the osmolarity of their microenvironment. In hyperosmolar conditions, *E. coli* represses the synthesis of larger porins (OmpF) while continuing to express smaller ones (OmpC) [32].

Mutations resulting in the loss of specific porins can occur in clinical isolates and determine increased resistance to beta-lactam antibiotics. Resistance to aminoglycosides and carbapenems emerging during therapy has also been associated with a lack of production of outer membrane proteins. In *P. aeruginosa*, resistance to imipenem appears to be due to an interaction between chromosomal beta-lactamase activity and a loss of a specific entry channel, the D2 porin [33].

The rate of entry of aminoglycoside molecules into bacterial cells is a function of their binding to a usually non-saturable anionic transporter, whereupon they retain their positive charge and are subsequently 'pulled' across the cytoplasmic membrane by the internal negative charge of the cell. This process requires energy and a threshold level of internal negative charge before significant transport occurs *(proton motive force)* [34]. These aminoglycoside-resistant isolates with altered proton motive force may occur during long-term aminoglycoside therapy. These isolates usually have a 'small colony' phenotype due to their reduced rate of growth.

Drug Efflux

Active efflux of antimicrobial agents is increasingly utilized by bacteria and fungi as a mechanism of antibiotic resistance. Some strains of *E. coli, Shigella*, and other enteric organisms express a membrane transporter system that leads to multidrug resistance by drug efflux [35]. Specific efflux pumps also exist that promote the egress of single classes of antimicrobial agents. Efflux mechanisms are the major mechanism of resistance to tetracyclines in Gram-negative bacteria. Some strains of *S. pneumoniae, S. pyogenes, S. aureus*, and *S. epidermidis*, use an active efflux mechanism to resist macrolides, streptogramins, and azalides [23]. This efflux mechanism is mediated by the *meF* (for macrolide efflux) genes in streptococci and *msr* (for macrolide streptogramin resistance) genes in staphylococci. A similar efflux system, encoded by a gene referred to as *mreA* (for macrolide resistance efflux), has been described in group B streptococci. This mechanism of resistance may be more prevalent in community-acquired infections than was generally appreciated. Dissemination of these resistance genes among important bacterial pathogens constitutes a major threat to the continued usefulness of macrolide antibiotics [36].

Active efflux mechanisms may also contribute to the full expression of beta-lactam resistance in *P. aeruginosa*. Multidrug efflux pumps in the inner and outer membrane of *P. aeruginosa* may combine with periplasmic beta-lactamases and membrane permeability components for full expression of antibiotic resistance [37]. Active efflux of fluoroquinolones by specific quinolone pumps or multidrug transporter pumps has also been detected in enteric bacteria and staphylococci [23].

Alter Target Sites

Resistance to a wide variety of antimicrobial agents, including tetracyclines, macrolides, lincosamides, streptogramins and the aminoglycosides, may result from alteration of ribosomal binding sites. The MLS_B-determinant has the genes that produce enzymes to dimethylate adenine residues on the 23-S ribosomal RNA of the 50-S subunit of the prokaryotic ribosome, disrupting the binding of these drugs to the ribosome.

Resistance to aminoglycosides may also be mediated at the ribosomal level. Mutations of the S12 protein of the 30-S subunit have been shown to interfere with binding streptomycin to the ribosome. Ribosomal resistance to streptomycin may be a significant cause of streptomycin resistance among enterococcal isolates. Ribosomal resistance to the 2-deoxystreptamine aminoglycosides (gentamicin, tobramycin, amikacin) appears to be uncommon and may require multiple mutations in that these aminoglycosides bind at several sites on both the 30S and 50S subunits of the ribosome [23].

Vancomycin and other glycopeptide antibiotics such as teicoplanin bind to D-alanine-D-alanine, which is present at the termini of peptidoglycan precursors. The large glycopeptide molecules prevent the incorporation of the precursors into the cell wall. Resistance of enterococci to vancomycin has been classified

as A–G based upon the genotype, type of target site modification and level of resistance to vancomycin and teicoplanin [38]. Strains of *E. faecium* and *E. faecalis* with high-level resistance to both vancomycin and teicoplanin have class A resistance. Class A resistance is mediated by the *vanA* gene cluster found on an R plasmid. This protein synthesizes peptidoglycan precursors that have a depsipeptide terminus (D-alanine-D-lactate) instead of the usual D-alanine-D-alanine. The modified peptidoglycan binds glycopeptide antibiotics with reduced affinity, thus conferring resistance to vancomycin and teicoplanin. The other classes of vancomycin resistance genes vary in level of resistance, species distribution and specific cell wall alterations [23, 38].

Vancomycin-intermediate strains of resistant *S. aureus* (VISA) have been isolated with heterogeneous resistance patterns. VISA strains express unusually thick peptidoglycan cell walls that are less completely cross-linked together. The cell wall in some strains of VISA contains non-amidated glutamine precursors that provide an increased number of false binding sites to vancomycin [39]. The vancomycin molecules are absorbed to these excess binding sites thereby reducing vancomycin concentrations at the growth point of peptidoglycan synthesis along the inner surface of the cell wall. The arrival of high level vancomycin resistance from vanA expressing *S. aureus* [17] has created a renewed sense of urgency in the need to develop novel strategies to combat multi-drug resistant bacterial pathogens.

Beta-lactam antibiotics inhibit bacteria by binding covalently to penicillin-binding proteins (PBPs) in the cytoplasmic membrane. These target proteins catalyze the synthesis of the peptidoglycan that forms the cell wall of bacteria. In Gram-positive bacteria, resistance to beta-lactam antibiotics may occur by a decrease in the affinity of the PBP for the antibiotic or with a change in the amount of PBP produced by the bacterium [23]. These low affinity binding PBPs may be inducible where their production is stimulated by exposure of the microorganism to the beta-lactam drug [40]. The structural gene *(mecA)* that determines the low-affinity PBP of MRSA shares extensive sequence homology with a PBP of *E. coli*, and the genes that regulate the production of the low-affinity PBP have considerable sequence homology with the genes that regulate the production of staphylococcal penicillinase [23].

The PBPs of beta-lactamase–negative penicillin-resistant strains of *N. gonorrhoeae, N. meningitidis*, and *Haemophilus influenzae* have shown reduced penicillin-binding affinity [41]. Their PBPs appear to be encoded by hybrid genes containing segments of DNA scavenged from resistant strains of related species, similar to penicillin-resistant pneumococci [23].

DNA gyrase (also known as bacterial topoisomerase II) is necessary for the supercoiling of chromosomal DNA in bacteria in order to have efficient cell division [23]. Another related enzyme, topoisomerase IV is also required for segregation of bacterial genomes into two daughter cells during cell division. These enzymes consist of two A subunits encoded by the *gyrA* gene and two B subunits encoded by the *gyrB* gene (or parC and parE for topoisomerase IV. Although spontaneous mutation ot the A- subunit of the *gyrA* locus is the most common cause of resistance to multiple fluoroquinolones in enteric bacteria, B-subunit alterations may also affect resistance to these drugs.

DNA gyrase (topoisomerase II) is the primary site of action in Gram-negative bacteria whereas topoisomerase IV is the principal target of quinolones in Gram-positive bacteria. Mutations in a variety of chromosomal loci have been described that resulted in altered DNA gyrases resistant to nalidixic acid and the newer fluoroquinolones in Enterobacteriaceae and *P. aeruginosa*. Many of these mutations involve the substitution of single amino acids at key enzymatic sites (located between amino acids 67–106 in the gyrase A subunit) that are involved in the generation of the DNA gyrase–bacterial DNA complex [42].

There are two common genes that mediate resistance to sulfa drugs in a wide variety of pathogenic bacteria. These are known as *sul1* and *sul2*. These genes give rise to altered forms of the target enzyme for sulfonamide, dihydropteroate synthase (DHPS) [43]. The altered DHPS enzymes mediated by the sulfonamide resistance genes no longer bind to sulfa yet continue to synthesize dihydropteroate from para-aminobenzoic acid substrate.

Trimethoprim is a potent inhibitor of bacterial dihydrofolate reductase (DHFR). A large number of altered DHFR enzymes with loss of inhibition by trimethoprim have been described from genes found primarily on R plasmids. These altered DHFR genes are widespread in Gram-negative bacteria and are also found in staphylococci (the *dfrA* gene) [44].

Protection of the Target Site

Tetracycline resistance may be mediated by a mechanism that interferes with the ability of tetracycline to bind to the ribosome. The ubiquitous *tetM* resistance gene and related tetracycline resistance determinants protect the ribosome from tetracycline action. The tetM gene generates protein with elongation factor-like activity that may stabilize ribosomal transfer RNA interactions in the presence of tetracycline molecules [45].

Excess Synthesis of the Inhibited Target

Sulfonamides compete with para-aminobenzoic acid to bind the enzyme dihydropteroate synthase, and thereby block folic acid synthesis necessary for nucleic acid synthesis. Sulfonamide resistance may be mediated in some bacteria by the over production of the synthetic enzyme dihydropteroate synthase. The gene responsible for DHPS is *felP* and strains of bacteria that produce excess DHPS can overwhelm sulfa inhibition [43]. Trimethoprim resistance may also occur in a similar fashion, by making excess amounts of dihydrofolate reductase from the bacterial chromosomal gene *folA* [44].

Bypass Mechanism of Resistance

An unusual mechanism of resistance to specific antibiotics is by the development of auxotrophs, which have specific growth factor requirements not seen in wild-

type strains. These mutants require substrates that normally are synthesized by the target enzymes, and thus if the substrates are present in the environment, the organisms are able to grow despite inhibition of the synthetic enzyme by an antibiotic. Bacteria that lose the enzyme thymidylate synthetase are 'thymine dependent'. If they can acquire exogenous supplies of thymidine to synthesize thymidylate via salvage pathways from the host, they are highly resistant to sulfa drugs and trimethoprim [23].

The Transmission of Resistance Genes Between Bacterial Species

Once an antibiotic resistance gene evolves, the resistance determinant can disseminate among bacterial populations by transformation, transduction, conjugation, or transposition. Favored clones of bacteria then proliferate in the flora of patients who receive antibiotics. Antibiotic-resistance genes were found among bacteria even in the pre-antibiotic therapy era [23]. However, selection pressures placed upon microbial populations by a highly lethal antimicrobial compound create an environment in which individual clones that resist the antibiotic are markedly favored. These resistant populations then proliferate and rapidly replace other susceptible strains of bacteria. While some antibiotic resistance genes place a metabolic 'cost' on bacteria, many microorganisms have evolved strategies to limit this cost by limiting expression, alternate gene products or phase variation. These mechanisms allow favorable but sometimes 'costly' genes that mediate antibiotic resistance to persist in the absence of continued antibiotic selection pressure and yet be rapidly expressed upon re-exposure to antibiotics [46].

Plasmids

Plasmids are particularly well adapted to serve as agents of genetic evolution and R-gene dissemination. Plasmids are extrachromosomal genetic elements that are made of circular double-stranded DNA molecules that range from less than 10 to greater than 400 kilobase pairs and are extremely common in clinical isolates of bacterial pathogens. Although multiple copies of a specific plasmid or multiple different plasmids, or both, may be found in a single bacterial cell, closely related plasmids often cannot coexist in the same cell. This observation has led to a classification scheme of plasmids based upon incompatibility groups [23].

Plasmids may determine a wide range of functions besides antibiotic resistance, including virulence and metabolic capacities. Plasmids are autonomous, self-replicating genetic elements that possess an origin for replication and genes that facilitate its stable maintenance in host bacteria. Conjugative plasmids require additional genes that can initiate self-transfer.

The transfer of plasmid DNA between bacterial species is a complex process, and thus conjugative plasmids tend to be larger than non-conjugative ones. Some small plasmids may be able to utilize the conjugation apparatus of a coresident conjugative plasmid. Many plasmid-encoded functions enable bacterial

strains to persist in the environment by resisting noxious agents, such as heavy metals. Mercury released from dental fillings may increase the number of antibiotic-resistant bacteria in the oral flora. Hexachlorophene and other topical bacteriostatic agents in the environment may actually promote plasmid-mediated resistance to these agents and other antimicrobial agents [47].

Transposable Genetic Elements

Transposons are specialized sequences of DNA that are mobile and can translocate as a unit from one area of the bacterial chromosome to another. They can also move back and forth between the chromosome and plasmid or bacteriophage DNA. Transposable genetic elements possess a specialized system of recombination that is independent of the generalized recombination system that permits recombination of largely homologous sequences of DNA by crossover events (the *rec*A system of bacteria). The *rec*A-independent recombination system ('transposase') of transposable elements usually occurs in a random fashion between non-homologous DNA sequences and results in whole-scale modifications of large sequences of DNA as a single event [23].

There are two types of transposable genetic elements, *transposons* and *insertion sequences.* These mobile sequences probably play an important physiologic role in genetic variation and evolution in prokaryotic organisms. Transposons differ from insertion sequences in that they mediate a recognizable phenotypic marker such as an antibiotic-resistance trait. Either element can translocate as an independent unit. Both elements are flanked on either end by short identical sequences of DNA in reverse order *(inverted repeats)*. These inverted-repeat DNA termini are essential to the transposition process. Transposons and insertion sequences must be physically integrated with chromosome, bacteriophage, or plasmid DNA in order to be replicated and maintained in a bacterial population. Some transposons have the capability to move from one bacterium to another without being transferred within a plasmid or bacteriophage. These *conjugative* transposons are found primarily in aerobic and anaerobic Gram-positive organisms and can rapidly and efficiently spread antibiotic resistance genes [30, 48].

Transposition, like point mutation, is a continuous and ongoing process in bacterial populations. Transposons are also essential in the evolution of R plasmids that contain multiple antibiotic-resistance determinants [47]. High-level vancomycin resistance (vanA) in enterococci is mediated by a composite transposon that encodes a series of genes needed to express vancomycin resistance [38]. Single transposons may encode multiple antibiotic-resistance determinants within their inverted-repeat termini as well [23].

Genetic exchange of antibiotic-resistance genes occurs between bacteria of widely disparate species and different genera. Identical aminoglycoside-resistance genes can spread between Gram-negative and Gram-positive bacteria and between aerobic and anaerobic bacteria [49]. Given the highly variable environmental selection pressures created by a wide variety of antibiotics and the plasticity of bacterial genomes, the ongoing evolution of multi-drug resistant bacterial organisms is probably inevitable [23].

DNA Integration Elements

The structural genes that mediate antibiotic resistance are often closely linked and may exist in tandem along the bacterial chromosome or plasmid. Genetic analysis of sequences of DNA adjacent to resistance genes has identified unique integration units near promoter sites [50]. These integration regions are known as *integrons*, and they function as convenient recombinational 'hot spots' for site-specific recombination events between largely non-homologous sequences of DNA. The integron provides its own integrase function [94] with a common attachment and integration site for acquisition of foreign DNA sequences.

Integrons are widespread in bacterial populations and provide a convenient site for insertion of multiple different resistance genes from foreign DNA sources. There are four classes of integrons with type I integrons being the most common in pathogenic microorganisms [23]. Integrons also serve as efficient expression cassettes for resistance genes. Integrons possess a promoter site in close proximity to the 5'end of the newly inserted DNA sequence. Numerous clusters of different resistance genes have been linked into integrons through specific insertion sites. Integrons may have as many as five resistance genes linked in sequence and flanked between specific 59 base-pair spacer units [50, 51]. Integron-mediated multiple resistance gene cassettes have been flanked by transposons, mobilized to plasmids, and then transferred between bacterial species by conjugation. By these systems of genetic exchange, widespread dissemination of multiple antibiotic resistance genes is accomplished in a rapid and frighteningly efficient manner [50].

Are We Approaching The End of the Antimicrobial Era?

For some time concerned scientists have been warning about the possibility of widespread antibiotic resistance leading to the loss of effectiveness of antibiotics in clinical medicine [49–52]. These warnings have largely been ignored as it was assumed that this human need and the profit motive of free enterprise would stimulate pharmaceutical companies to continuously develop new antibiotics. If we could discover new targets for future antimicrobial drugs it may be possible to keep pace or even exceed the rate of antibiotic resistance gene development by microbial pathogens. For a number of disconcerting reasons, humans may be losing ground rather than gaining on pathogens in the 21st century.

A recent survey of new pharmaceutical products in 2002 found only five new antibiotics out of the 506 new molecular entities in the research and development pipeline [52]. The pace of new antibiotic discovery is turning into a trickle and drying up compared to what it was even 20 years ago [53]. The market reality is regrettably set against the development of new antibiotics in favor of more lucrative options with greater market profit from drugs for chronic illnesses with less risk and longer revenue streams [52–54]. The reimbursement and return on investments are unfavorable for antibiotics and the market system is not meeting the needs of society with respect to new antibiotic development. Some far reaching and bold initiatives are desperately needed if a crisis in loss of antibi-

Table 4. Disincentives for new antibiotic drug development

Disincentives	Possible Solutions
Expense (800 million US dollars/ new molecular entity) forces companies into broad-spectrum antibiotic market	Shorten regulatory process; extend patent life; government protection from liability claims; speed development with genomics and high throughput screening process
Specific narrow-spectrum antibiotics are small markets – 'niche' product – difficult to regain development costs	Not-for-profit drug companies; government funding of small market-narrow spectrum drugs; move research and development to low income status countries
Restricted use and short duration of treatment limits profits (antibiotic treatment is for days-weeks not years)	Improve patent position of antibiotics; extend 'orphan' drug status to new antibiotics
Antibiotic resistance development limits lifespan of drug market	Ban non-medical use of antibiotics; good antibiotic stewardship
Regulatory difficulties with combination therapies (must test each individual component first before combination)	Change regulatory requirements; not-for-profit companies with multiple partners; anti-trust law exemptions
Use of 19th century diagnostic methods (culture and susceptibility tests) to treat 21st century diseases encourages empiric broad-spectrum antibiotic drug use	Employ real-time PCR, genomics, proteomics to identify pathogens, and resistance genes; target and treat specific infections with narrow spectrum drugs PCR: polymerase chain reaction

otic effectiveness is to be avoided [52, 53]. The disincentives for new antibiotic development and some proposed solutions are listed in Table 4.

The Future of Antibiotic Use in Clinical Medicine

Bacterial strains contain complex aggregations of genes that may be linked together to combat the inhibitory effects of antibiotics. Since prokaryotic organisms all contribute to a common 'gene pool', favorable genes mediating antibiotic resistance may disseminate among bacterial diverse microbial genera and species. Increasing evidence of multiple antibiotic resistance mechanisms within the same bacterium against a single type of antibiotic, and cooperation between bacterial populations within biofilms attest to the remarkably ingenuity and flexibility of bacterial populations [23, 29, 30]. Thus the use of one antibiotic may select for the emergence of resistance to another. Mobile genetic elements and rapidly evolving integron cassettes with multiple antibiotic resistance genes endow bacteria with a remarkable capacity to resist antibiotics [50]. Although the development of antibiotic resistance may be inevitable, the rate at which it develops can be reduced by the rational use of antibiotics.

The wider accessibility to molecular techniques and computer technology to rapidly identify the specific microorganisms, their resistance potential, and track their spread between patients within the hospital and or the community will be of considerable benefit in the control of antibiotic resistance. The need to utilize empiric, broad-spectrum antibiotics for days and even weeks while samples are being sent for culture and susceptibility testing needs to stop. We need specific information in real time to assure patients with specific infections are being treated with effective, narrow-spectrum drugs [23].

The use of antibiotics for non-medical uses should be entirely banned. Up to 50% of antibiotic use today is for non-medical use in agriculture, food preparation, and other industrial uses [52]. This adds to environmental contamination with low levels of antibiotics. Sub-inhibitory concentrations of antibiotics foster the development of resistant clones of bacteria that can cause infections in humans. The use of non-antibiotic approaches to the management of infectious diseases needs to be supported and developed. The use of plasma-based antibody therapies and anti-bacterial, anti-viral and anti-fungal vaccines should be encouraged in the future [55–57].

The management of common invasive pathogens such as staphylococcal infections has become very complicated given the rapid spread of simultaneous beta-lactam, aminoglycoside, and quinolone-resistant isolates [58]. Recent reports of vancomycin-resistant *S. aureus* in Japan and the United States suggest that common, invasive, microbial pathogens may become refractory to any chemotherapeutic agent in the future [17, 23, 58].

New drug discoveries have allowed us to be one step ahead of the bacterial pathogens for the latter half of the twentieth century. It is unlikely we will continue this record of remarkable success against microbial pathogens in the new millennium. The rapid evolution of resistance has limited the duration of the effectiveness of antibiotics against certain pathogens. The best hope for the future is the continued development of new antibiotic strategies [53]. In order to retain the antimicrobial activity of existing and new antibiotics, clinicians can assist through careful antibiotic stewardship and tightened infection control measures. Antimicrobial agents have had a substantial impact in decreasing human morbidity and mortality rates and have served us well over the antimicrobial era. It behooves us to improve our diagnostic and surveillance efforts and to exercise caution in administering antibiotics if we are to maintain their continued efficacy.

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