EMBO reports

viewpoint

Microbes have the last word

A drastic re-evaluation of antimicrobial treatment is needed to overcome the threat of antibiotic-resistant bacteria

Julian Davies

Many organisms have evolved by interacting with one another through one or more of three mechanisms: commensalism (co-existence), symbiosis (collaboration) or parasitism (confrontation). Human evolution is no exception; we have evolved in the presence of large numbers of microbes including commensals and symbionts that are vital to our survival. Nevertheless, of all the microbial species inhabiting the biosphere, a small but deadly proportion is pathogenic to humans and animals.

Throughout history, infectious diseases in humans have been observed, treated and recorded. More than 100 years ago, Ferdinand Cohn, Louis Pasteur and Robert Koch collectively discovered that microbes—including bacteria, fungi and viruses—are the causative agents of infection. In fact, the period between 1890 and the 1930s, when the main human pathogens were isolated and identified, can be regarded as the golden age of microbiology; finally, the enemy was known.

Although these discoveries did not lead to major advances in the treatment of disease, scientists began to develop and use the first crude vaccines against bacterial and viral infections such as typhus, yellow fever, tetanus and diphtheria. Furthermore, a search began in earnest for a 'magic bullet' that could cure disease by specifically targeting and killing microbes. This began with the use of chemicals, previously used to stain tissues, to deliver toxins harmful to specific bacteria. In the early 1900s, pioneering research into the use and modification of synthetic chemotherapeutic agents by Paul Ehrlich's laboratory led to the discovery of Salvarsan® (arsphenamine), an anti-syphilitic agent introduced as the first—albeit unpleasant—antimicrobial therapy.

However, until the mid-1930s, the treatment of bacterial infections was largely empirical and still relied on native and cultural variations of snake oils and elixirs. Nonetheless, an understanding of good patient care developed along with markedly improved public hygiene and sterile procedures. In 1932, Gerhard Domagk synthesized the first active sulphonamide (Hager, 2006), and this work was followed by studies in the UK and France that led to the successful introduction of sulphonamides for antibacterial therapy in 1938. Since then, many sulphonamide derivatives have been made, and they continue to be used for the treatment of various bacterial diseases.

Any septuagenarians today will have experienced the change from a world in which infection was left untreated and often resulted in mortality, to a world in which antibiotics and vaccines can be used to control the spread and progression of most diseases

The forerunners of current antibiotics were penicillin, a fungal product discovered by Alexander Fleming in 1928 but not introduced for human use until 1942, and streptomycin, a bacterial product isolated by Selman Waksman's laboratory in 1944 and introduced for treatment shortly thereafter. Both compounds are still in extensive use today, but for most applications in industrialized nations, more effective, often oral, antibiotics have replaced them.

This rapid development of antimicrobial compounds during the past 100 years has vastly improved the treatment of infection and disease. Penicillin is estimated to have made a significant contribution during the Second World War, markedly reducing the number of lives lost by Allied Forces to infections associated with the amputation of limbs or shrapnel wounds. Any septuagenarians today will have experienced the change from a world in which infection was left untreated and often resulted in mortality, to a world in which antibiotics and vaccines can be used to control the spread and progression of most diseases. But are we now facing an inevitable return to the pre-antibiotic era?

When penicillin, streptomycin and the sulphonamides became widely available, many thought that all pathogenic microbes would succumb and infectious disease would be a thing of the past. Antibiotic resistance was not considered a problem, despite the fact that microbiologists had already isolated mutants of *Escherichia coli* and other bacteria resistant to antibiotics in the laboratory. Even the development of streptomycin resistance by *Mycobacterium tuberculosis* during a course of treatment was not recognized as an omen of what was to come. Instead, the 1950s saw the rise of a pharmaceutical industry that created potent new antimicrobials such as chloramphenicol, erythromycin and tetracycline. The ability of these agents to rapidly and effectively kill a wide variety of infectious bacteria was a medical revolution and established a belief in the all-encompassing power of antibiotics.

However, in the early 1950s, there were disturbing reports from Japan describing

an epidemic of dysentery caused by strains of *Shigella* that rapidly developed antibiotic resistance (Fig 1). In the late 1950s, Japanese scientists reported that resistance to multiple antibiotics not only developed quickly and simultaneously, but also seemed to transfer from resistant to sensitive strains. Most bacterial geneticists in Europe and the USA met the latter claim with disbelief—the prevailing evidence at the time was that resistance was specific and occurred by mutation at low frequency. Early publications of the phenomenon appeared in Japanese journals but were not made available elsewhere. In fact, attempts to publish these findings were rebuffed by the infectious diseases community.

Similarly, the identification of multi-drug resistant bacteria by scientists in the UK and Germany in the early 1960s was met with denial and scorn. Eventually, the Japanese work was published in Western journals and the phenomenon was considered to be validated after transferable resistance was identified in the USA in 1966 (Davies, 1995). Even then, the impact of this finding was not appreciated—it took more than a decade for physicians and scientists to realize that transferable antibiotic resistance was responsible for the increasing problems of infection in hospitals and subsequently in the community.

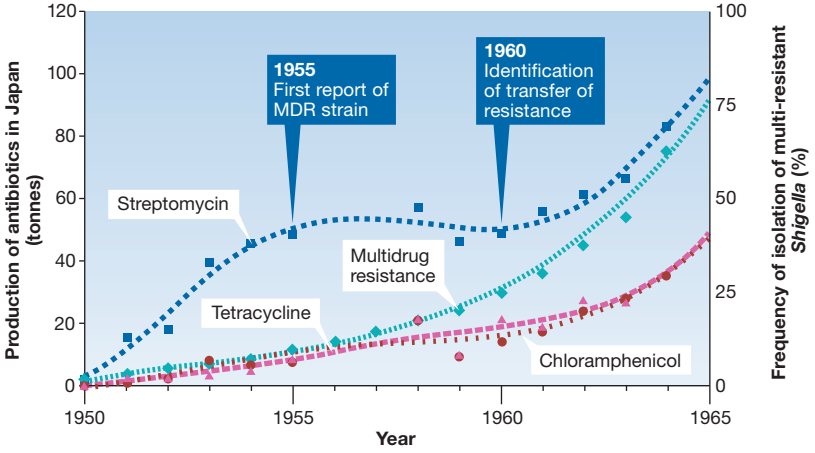

Fig 1 | The relationship between antibiotic-resistance development in *Shigella* dysentery isolates in Japan and the introduction of antimicrobial therapy between 1950 and 1965. In 1955, the first case of plasmid-determined resistance was characterized. MDR, multidrug resistance.

Unfortunately the end result of modifying existing antibiotics to make them completely refractory to any resistance mechanism is compounds that have no antibiotic activity at all

This is just one example in a long history of scientists and physicians who have underestimated the power of microbes. A second example is the early assumption that mutations that increase resistance conversely weaken the organism, causing it to be outcompeted by wild-type strains. Although the acquisition of resistance genes frequently comes at a cost to fitness, especially when a plasmid is transferred, antibiotic-resistant pathogens rapidly acquire compensatory mutations that restore their full competitive fitness and virulence (Fig 2). This is not surprising, given that most natural bacterial isolates stably maintain one or more plasmids encoding genes to metabolize or otherwise neutralize toxic compounds, and that specific mechanisms have evolved to maintain extrachromosomal elements in cells (Moritz & Hergenrother, 2007).

Another misleading idea was that the use of synthetic antimicrobials would be less prone to plasmid-determined resistance. Regrettably, the discovery of transferable resistance to sulphonamides and trimethoprim rapidly ended this pipe dream, and recent work with the fluoroquinolone (FQ) antibiotics has dealt a double blow to the assumption. It was predicted that resistance to FQ would be rare in the clinic because pathogens would need multiple mutations to become resistant; however, highly FQ-resistant strains are quickly becoming commonplace. There was also the hope that FQ resistance would not be transferable—but it is now increasing, owing to two different and completely unexpected plasmid-encoded biochemical mechanisms (Robicsek *et al*, 2006). The first was the acquisition of the quinolone resistance (*qnr*) gene encoding a protein with pentapeptide repeats that protects DNA; the second came through mutations in a common acetylating enzyme, such that FQs became modified as new substrates. Antibiotic resistance is now endemic worldwide and has serious negative effects on therapy (Levy & Marshall, 2004).

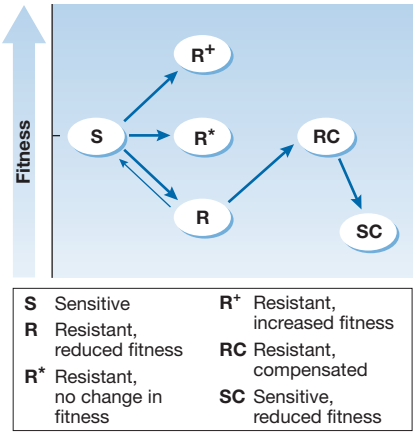

Fig 2 | The biological cost of antibiotic resistance and its genetic compensation.

The initial response of the pharmaceutical industry was to search for semi-synthetic derivatives of current antibiotics that would be refractory to resistance mechanisms. Antibiotics such as the β-lactams (penicillins) were subjected to a series of chemical modifications to generate 'novel' derivatives that were not substrates for existing resistance mechanisms in pathogens. This 'keeping up with the bugs' approach has led to generations of 'improved' antibiotics, such as methicillin and related β-lactams, which have been successful against the extant organisms for limited periods of time.

However, the natural occurrence of microbial gene 'juggling' quickly countered new drug developments. The much-feared methicillin-resistant *Staphylococcus aureus* (MRSA) is a prime example of the ability of a microbe to out-mutate human

ingenuity. The past 40 years or so has seen a resistance 'arms race' between pharmaceutical companies and bugs, particularly in the case of broad-spectrum antibiotics such as β-lactams, aminoglycosides and fluoroquinolones. However, as Louis Pasteur once purportedly said, the microbes always have the last word. Unfortunately the end result of modifying existing antibiotics to make them completely refractory to any resistance mechanism is compounds that have no antibiotic activity at all.

Most of the agents commonly used today were discovered in the 1950s and 1960s. Since then, the pharmaceutical industry has been largely occupied with improving or extending the activity of existing compounds. The search for new antibiotics has continued, but less vigorously. In the 1990s, research into bacterial genomics triggered a resurgence of drug discovery. Significant investments were made in genome sequencing and high-throughput screening as the new tools for identifying new antibiotics. Both scientists and the managements of pharmaceutical companies thought that genomic analyses of bacterial strains would provide new drug targets that could be screened against large libraries of synthetic organic compounds to identify specific inhibitors. This was an expensive failure. Although many enzyme inhibitors were identified, few actually inhibited bacterial growth and, of these few active compounds, it seems that only one has made it to clinical testing (Payne *et al*, 2007).

As noted by the distinguished ecologist Edward Wilson, microbial diversity is beyond practical calculation

There are several reasons for this failure. Among them are the inability of bioinformatics to identify target genes, and the use of synthetic chemicals that work in the test tube but do not inhibit cell growth. One unfortunate outcome was that each pharmaceutical company independently sequenced the genomes of numerous pathogens—the usual response in an effort to stifle competition—resulting in an enormous duplication of closely guarded genetic information that was not made available to the wider academic community for basic research.

This forced university laboratories and forward-thinking organizations—such as the Sanger Centre (Hinxton, UK), The Institute for Genomic Research (Rockville, MD, USA) and the Joint Genome Institute (Walnut Creek, CA, USA)—to repeat all the sequencing and make highly accurate sequences of the genomes of most major pathogens publicly available. As of July 2007, more than 500 prokaryotic genome sequences will have been completed. The analysis of bacterial genomes has already led to important discoveries about the crucial role of horizontal gene transfer and genomic islands in the evolution, expression and development of virulence functions. However, whether this mass of pathogenomic information will stimulate new efforts in antimicrobial drug discovery remains to be seen.

One thing that is certain is that the identification of complex metabolic networks, and the development of sophisticated bioinformatic and genetic tools should generate enormous amounts of information about microbes and their hosts. These efforts will be far more productive than those of the previous decade, and the information more usefully applied to the development of therapeutic and preventive alternatives to existing antibiotics and vaccines.

Current accounts of antibiotic resistance in the scientific literature are indicative of a new and growing problem because they almost exclusively refer to 'new', 'novel' or 'evolving' resistance mechanisms. In fact, mutation or the acquisition of a gene or genes are the only two known genetic mechanisms that enable a microbe to evade an antibiotic, toxin or any other inhibitory chemical substance. Around ten biochemical mechanisms of resistance are already known (Table 1) and many more can be expected. The range of genetic 'jugglery' and the metabolic dexterity of microbes seems to be infinite (Depardieu *et al*, 2007).

Table 1 | The biochemical mechanisms of antibiotic resistance. Those mechanisms shown on the left are usually acquired by mutation; however, all of the mechanisms shown can be acquired by plasmid-associated gene transfer

Increased efflux of antibiotic	Enzymatic inactivation of antibiotic
Decreased influx	Sequestration of antibiotic
Target modification	Target bypass
Target amplification	Protection of target
Repair of damaged target	Intracellular localization
Biofilm formation	—

Mutation and gene acquisition frequently occur together. Mutation is easily shown and can be traced by epidemiological analyses; it is likely that mutations that afford early low-level resistance are the forerunners of high-level multi-antibiotic resistance, at which time gene acquisition comes into play (Baquero, 2001). The million-dollar questions then are: how, when and from where are resistance genes acquired?

Fifty years of exposure to massive amounts of antibiotics is just a small hiccup in microbial history

Many clinically important pathogens are 'naturally' less susceptible to antibiotics. For example, *M. tuberculosis* is resistant to penicillins and erythromycin, and *Pseudomonas aeruginosa* shows intrinsic resistance to many antibiotics including the sulphonamides and β-lactams. The reason might be that pseudomonads are denizens of many hostile environments—they metabolize fuel oil, for example—and can probably survive these conditions because of their ability to pump out various toxic organic compounds, including antibiotics (Piddock, 2006). Over time, several useful inhibitors for the pseudomonads and other multidrug-resistant pathogens have been discovered, but these too have been rendered less effective as plasmid-determined mechanisms of resistance have evolved. In hindsight this was to be expected, as plasmids and other extrachromosomal elements are common in environmental microbes.

As antibiotic-resistance plasmids were undetectable in pre-antibiotic collections of bacteria, it is generally assumed that they are of recent origin. In 1983, scientists examined a collection of some 200 clinical *Enterobacteria* isolates, collected between 1920 and 1930, for the presence

of antibiotic-resistance genes and gene-transfer mechanisms. They found plasmids, but no transferable antibiotic resistance (Datta & Hughes, 1983; Hughes & Datta, 1983). Presumably these resistance mechanisms did not evolve *de novo*—they must have genetic precursors in environmental microbes. In 1973, biochemical studies identified that Streptomycetes—the bacteria responsible for producing the majority of existing antibiotics—have enzymes that modify and inactivate antibiotics (Benveniste & Davies, 1973). More recently, extensive shotgun sequencing of microbial genomes has revealed orthologues of known resistance genes in many pathogenic and non-pathogenic bacteria. These genes are close relatives of the antibiotic-resistance genes present on R-plasmids and found as chromosomal genes in many bacterial strains. In current parlance, this environmental reservoir of resistance genes is referred to as the resistome (D'Costa *et al*, 2006).

Although our understanding of the natural sources of resistance genes is increasing, their actual biochemical functions remain largely unknown. This is but one small aspect of the larger problem of our rudimentary understanding of the biological complexity of microbial populations in nature. As noted by the distinguished ecologist Edward Wilson, microbial diversity is beyond practical calculation. Or, to paraphrase Douglas Adams, the author of *The Hitchhikers Guide to the Galaxy*: microbial space is big. You just won't believe how vastly, hugely, mind-bogglingly big it is.

However we describe them, the precursors of known antibiotic-resistance genes can be found in vastly different environments and in diverse microbial populations. Most antibiotics are natural microbial products that are presumed to act as competitive or signalling agents in inter-microbial community networking. In either case, modulation of their effects could occur by structural modification or other biochemical changes mediated by 'resistance' mechanisms.

Fifty years of exposure to massive amounts of antibiotics is just a small hiccup in microbial history. In the 200–300 years since the beginning of the industrial revolution, humankind has polluted the biosphere with toxic organic molecules at an unprecedented rate. Antibiotics are just a recent addition and microbes have continued to survive and are flourishing (de la Cruz & Davies, 2005).

The main causes of microbial recalcitrance to any toxic agent—be it antibiotics, disinfectants, polychlorinated aromatics, heavy metal derivatives or biocides—are efflux pumps that lower the intracellular concentration of any foreign compound by pumping molecules out of the cytoplasm. The genes for efflux systems show strong evolutionary conservation, come in various shapes, sizes and properties, and provide the basis for nutrition and/or protection. They might also have narrow or broad molecular specificity. For example, the *mex* family of efflux pumps is a widely distributed system that can rid the cell of a range of environmental chemicals, including antibiotics (Groh *et al*, 2007).

Obviously, most plasmid-borne resistance genes found in bacterial pathogens originate in environmental microbes. From exactly which environment and which microbe is more difficult to establish—and how they found their way into the pathogen in the first place is a matter of speculation. However, there are various mechanisms of resistance acquisition and horizontal gene transfer—any one of which will suffice. Transduction, conjugation and transformation are the best-known and most important mechanisms of gene pick-up and transfer in bacteria, although other mechanisms such as cell fusion might occur in nature. Gene transfer is a natural, constant but random process and it is therefore difficult to identify the immediate precursor of any new gene. The isolation of a plasmid-containing strain in the laboratory tells us nothing about where the plasmid and its associated genes came from.

…the molecular origins of the antibiotic-resistance gene cassettes remain a mystery

In 1989, the discovery of integrons—genetic structures that are able to acquire, exchange and express 'gene cassettes' needed for specific biochemical functions—provided a possible mechanism of gene pick-up (Stokes & Hall, 1989). It is now clear that the plasmid-mediated resistance discovered in Japan in the 1950s involved resistance gene clusters generated by integron mechanisms. As previously mentioned, there is no good evidence for the presence of integron elements before the 1950s, but they must have been somewhere in some form.

Resistance integrons are the established mechanism by which Gram-negative bacteria acquire resistance genes. However, they have also recently been isolated in Gram-positive bacteria—this primitive chemical distinction is no barrier to horizontal gene transfer. Integrons themselves are not transmissible, but are associated with various vectors such as bacteriophages, plasmids and transposable elements, which are frequently integrated into bacterial chromosomes. There are three main integron types based on their integrase sequences: class 1 and class 2 integrons are the most common, whereas class 3 are rare and do not appear to be significant carriers of antibiotic-resistance genes—at least, not the known ones (Fluit & Schmitz, 2004; Fig 3).

More than 100 antibiotic-resistance gene cassettes have been identified in surveys of integron-associated genes and include all main classes of antibiotics. Metagenomic studies of bacterial populations in the environment continuously uncover new cassettes, although the vast majority of these do not encode known antibiotic resistance—in fact they do not encode any known function (Nemergut *et al*, 2004; Stokes *et al*, 2001). The existence of these cassettes implies that they are associated with integron clusters and might encode proteins that are important for bacterial community ecology (Mazel, 2006; Michael *et al*, 2004). The study of this genetic treasure trove is in its infancy and has much to reveal about the role of gene cassettes in genome evolution; however, the molecular origins of the antibiotic-resistance gene cassettes remain a mystery.

Infections by microbial pathogens that are resistant to almost all available antibiotics are becoming more common throughout the world. The rise in antibiotic resistance has contributed to an increasing toll in human morbidity and mortality. Pathogens such as MRSA—formerly inhabitants of intensive care units—have moved into the non-hospital community, concomitantly evolving to become more transmissible and a cause of much concern (Bloomfield, 2006).

We urgently need novel antimicrobial agents; however, there are few new antibiotics on the horizon or in the research pipeline. The medical communities in North America and Europe have recognized this dire situation and called for government action to

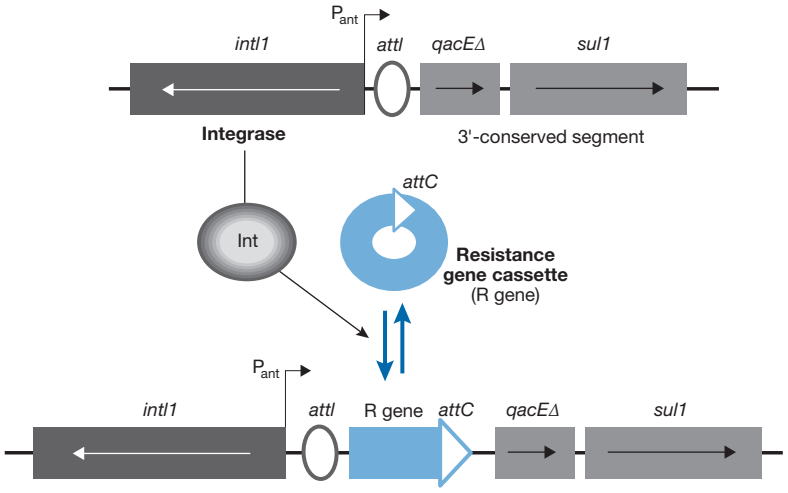

Fig 3 | The integron mechanism of antibiotic-resistance gene cassette acquisition. The integron integrase is responsible for the integration of circular-resistance gene cassettes. It does this by catalysing site-specific recombination between the att sites on the gene cassette and the plasmid. Cassette clusters are assembled in linear arrays. (Drawing provided by P. Courvalin.) AttC, attachment site (cassette); attI, attachment site (integron); intI1, integrase 1; P_{ant}, promoter for gene cassette expression; qac, quaternary ammonium compound resistance; sul, sulphonamide resistance.

encourage discovery efforts by the pharmaceutical industry. It is surprising that such incentives should be necessary to develop treatments for sick people, but one reason is that the current costs of discovery research, pharmaceutical development and clinical trials for a new antibiotic are about US$1 billion, which can rarely be recovered in the present regulatory and commercial climate (Nathan & Goldberg, 2005; Projan, 2003).

Almost any new compound that makes it through regulatory approval will be put on a restricted list to be used only when other antibiotics have failed, thereby limiting its market. For a publicly traded pharmaceutical company it comes down to a marketing decision. Most current 'lifestyle' drugs are highly profitable, whereas antimicrobial agents are not. Despite the fact that infectious diseases remain the leading cause of mortality worldwide, the leaders of the pharmaceutical industry have nonetheless cut back in a losing market, which leaves small biopharmaceutical companies and academia to search for new therapeutics.

At a time when leading physicians are speaking of 'the threat of untreatable pathogens' and 'a return to the pre-antibiotic era', the withdrawal by pharmaceutical companies from the field of antibiotic discovery has been met with shock and dismay. Antibiotics have been the main component of infectious disease therapy since the 1950s and, despite all the pessimism, they remain effective under most conditions. Responsible groups in the USA and Europe, in government, academia, medicine and industry have proposed a series of measures to address the lack of new therapeutics, and the need to contain the increase and spread of resistant strains and resistance genes (Spellberg *et al*, 2004; Talbot *et al*, 2006; Finch & Hunter, 2006). These include providing incentives to industry in the form of extended patent protection and other encouragements to stimulate research in antibiotic discovery. Transferring more of the early discovery research to publicly funded academic laboratories and small biopharmaceutical companies could be another way to promote the discovery of new therapies.

These proposals also emphasize the need for alternatives to antibiotics such as vaccines, immunomodulatory agents, probiotics and even bacteriophage therapy should all be considered. This last alternative has been shown to be effective under some conditions and is now being revived (Chibani-Chennoufi *et al*, 2004). More importantly, the recommendations call for improved diagnostics, dosing and surveillance in the clinic to control the development and spread of resistance. Obviously this must be accompanied by more basic research on the biology of disease and the development of resistance. Many believe that the future depends on the use of antibiotics with site-specific, narrow-spectrum activity prescribed on the basis of accurate and rapid diagnostic methods. The use of such 'niche' drugs, instead of broad-spectrum agents, would allow better identification and management of resistance outbreaks. The indiscriminate use of broad-spectrum antibiotics has contributed significantly to the antibiotic-resistance crisis. All antibiotic treatments must be controlled to the extent that physicians prescribe them accurately and only when needed.

Antibiotic-resistant cholera, tuberculosis, intestinal infections, and parasitic and viral infections are essentially pandemic because of antibiotic over-use without the control of prescription and dosage. A practical solution would be to produce effective vaccines; however, it would require a lot of research to develop vaccines against the main microbial pathogens. With a few notable exceptions—such as hepatitis B—vaccine development is a slow and difficult task. For example, we still do not have an effective vaccine against HIV despite significant research and investment. Designing vaccines for bacterial infections is even more problematic.

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Another continuing concern is the misuse of large quantities of antibiotics in agriculture and aquaculture for promoting growth and other non-therapeutic goals. Despite a ban on the use of antimicrobials in animal feeds in Europe, about half of the antibiotics produced worldwide are for non-human use. Consequently, serious outbreaks of food-borne disease by multidrug-resistant *E. coli*, *Salmonella typhimurium*, *Campylobacter jejuni* and *C. coli* through contaminated vegetables, eggs, poultry and meat are becoming more common worldwide.

The problem of resistance accompanies the use of any therapeutic agent. Resistance is inevitable, but it can be managed by the well-regulated use of appropriate treatments

in addition to a pipeline replete with alternative drugs and therapies. And there are more simple measures: to limit the spread of a multidrug-resistant infectious organism, all we have to do is heed the advice that Ignaz Semmelweis gave two centuries ago: the vigorous practice of good hygiene such as the thorough washing of hands does wonders.

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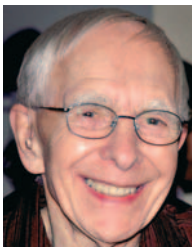

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