Who Will Win the Fight?

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Use of antibiotics in the last 70 years has transformed human health. With them we can survive bacterial infections that routinely killed and disabled our ancestors. But without new antibiotics, we may soon be exposed once more. Jeremy Garwood reports on the inexorable rise of multidrug-resistant bacteria, some of which may already be resistant to everything we have.

February 12th, 1941, a police constable, Albert Alexander, was the first person to be clinically treated with the antibiotic drug, penicillin. He had accidentally scratched his mouth on a rose thorn two months earlier. The scratch became badly infected with *Staphylococcus* and *Streptococcus* bacteria. In hospital, doctors tried to treat him but his head was covered with abscesses – they had to cut out one of his eyes to prevent the infection spreading further.

In 1938, Howard Florey, Professor of Pathology at Oxford University, began research on penicillin, an antibacterial agent produced by mould that had been reported in 1929. Wartime Britain two years later was faced with possible German invasion, so Florey decided to join the war effort, converting all his lab's resources to cultivating mould and purifying penicillin.

A miracle drug

By 1941, he had enough to test on a human volunteer. Worried that a drug with antibacterial properties might also be toxic to people, Florey chose a patient in a terminal condition. Constable Alexander was given an intravenous infusion of 160mg of penicillin. Within 24 hours, his temperature had dropped, his appetite had returned and the infection had begun to heal. However, Florey's laboratory only had a small quantity of penicillin (they were even obliged to re-extract it from their patient's urine). After four days treatment, Alexander was well on the way to recovery. Then the stock of penicillin ran out. He died a month later.

Florey took note and switched to sick children, who required smaller quantities of penicillin. He soon demonstrated the efficacy of his 'miracle drug'. The 'Age of Antibiotics' was born. Penicillin was soon followed by streptomycin (1944), chloramphenicol (1947), cephalosporins (1948), etc. For the first time in human history, the ever-present threat of bacterial infection had been mastered. Antibiotics cured not only acute lethal infections, such as septicemia (blood poisoning), meningitis and pneumonia but also disabling ones such as chronic infections of the sinuses, joints and bones.

Resistance

However, from the moment antibiotics were first used, bacteria began to appear that were resistant to them. In 1946, a year after the widespread use of penicillin, several strains of *Staphylococcus aureus* had already become resistant to it.

In fact, each time a new antibiotic has been used, bacteria have developed resistance. By the 1970s, there were penicillin-resistant *Neisseria gonorrhoeae* (PPNG), and beta-lactamase-producing *Haemophilus influenzae*. Methicillin-resistant *Staphylococcus aureus* (MRSA) and the resurgence of multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB) appeared in the late 1970s. The 1980s and 1990s saw resistance in strains of common enteric and non-enteric Gram-negative bacteria such as *Shigella*, *Salmonella*, *Vibrio cholerae*, *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

In 2010, at least 440,000 cases of multidrug-resistant tuberculosis were detected in 69 countries, resulting in around 150,000 deaths. In the EU, there are currently around 25,000 people a year who die of multidrug-resistant infections, two-thirds of which were caught while hospital patients. In the US, the situation may be even worse, with 17,000 deaths each year from antibiotic-resistant MRSA alone.

Resistant bacteria from hospitals are also causing more 'community-acquired' infections, e.g. strains of *Streptococcus pneumoniae*, *Staphylococcus aureus*, and vancomycin-resistance Enterococci (VRE). Not only can these cause serious infections that are increasingly difficult to treat with an effective antibiotic but some resistant bacteria have also acquired toxins that make them more virulent. For example, strains of MRSA that express Panton-Valentine leukocidin, a cytotoxin, which causes necrotic lesions in soft tissue that can kill patients in 72 hours. And the serious food poisoning outbreak in Germany in May 2011 (initially blamed on Spanish cucumbers) was

caused by a novel strain of *E. coli* O104:H4. In addition to multiple antibiotic resistance genes (including 5 beta-lactamases!), this strain had acquired two copies of the Shiga toxin stx2 prophage gene cluster, a toxin that can cause bloody diarrhea and hemolytic-uremic syndrome. By the end of July, there had been at least 45 deaths and several thousand hospitalisations. The infection was traced back to salad sprouts from a German farm but it's still not clear where it originally came from.

Are We in the Post-Antibiotic Era?

This was the question posed in 2005 by Alfonso Alanis, Vice President of Lilly Research Laboratories (*Arch Med Res,* 36:697–705). Alanis described the worrying conjuncture of bacteria developing resistance against all known classes of antibiotic at a time when pharmaceutical companies were losing interest in discovering new ones. He pointed to increasing evidence that certain medical and agricultural practices had considerably speeded-up the rate at which antibiotic resistance was developing. Particularly worrying was the appearance of the 'superbugs', bacterial strains that could simultaneously survive exposure to several different classes of antibiotic. What happens when there are no more antibiotics left to treat such infections?

For World Health Day 2011, the World Health Organisation (WHO) chose the theme: 'Combat Drug Resistance.' Margaret Chan, WHO's Director-General, said, "We are now on the brink of losing this precious arsenal of medicines. The use and misuse of antimicrobials in human medicine and animal husbandry over the past 70 years have increased the number and types of microorganisms resistant to these medicines, causing deaths, greater suffering and disability. If this phenomenon continues unchecked, many infectious diseases risk becoming uncontrollable. In the absence of urgent corrective and protective action, the world is heading towards a post-antibiotic era, in which many common infections will no longer have a cure."

Greater urgency has been given to the WHO's call by the discovery of an antibiotic resistance gene in 'superbugs' that may already be resistant to every known antibiotic.

In 2008, Timothy Walsh, who works on antibacterial agents and the genetics of resistance at Cardiff University, was asked by Stockholm's Karolinska Institute to look at a strange new bacterium isolated from the urine of a man repatriated from a hospital in New Delhi, India. It was *Klebsiella pneumoniae*, one of the most frequent causes of pneumonia and bloodstream infection in hospitalised patients. But this strain contained a new gene that rendered the *Klebsiella*, which was already multidrug-resistant, insensitive to the only remaining group that worked reliably and safely – the carbapenems, the 'drugs of last resort'. Walsh identified the enzyme coded by the gene as a metallo-beta-lactamase and named it after New Delhi – NDM-1 (*Lancet Infect Dis*, 2010, 10:597-602).

Carbapenems are a class of beta-lactam antibiotics capable of killing most bacteria by inhibiting the synthesis of one of their cell wall layers. They were specifically developed to overcome antibiotic resistance mediated by beta-lactamase enzymes and were first used in the 1980s. Inevitably, carbapenemase enzymes have appeared that inactivate carbapenems in resistant bacteria. These enzymes are considered particularly dangerous because they can hydrolyse a wide range of different antibiotics. Another carbapenemase, *Klebsiella pneumoniae* carbapenemase (KPC), first detected in US hospitals in 1996, has since spread worldwide despite vigorous medical control measures.

NDM-1 – the beginning of the end?

NDM-1 is a metallo-beta-lactamase (MBL). These carbapenemases typically have an active site containing two zinc ions that coordinate and present polarised water ions for the oxyanion attack on the beta-lactam ring. This method of attack and hydrolysis is unique among beta-lactamases and it is clinically critical since the enzyme does not form a stable, or even pseudo-stable, covalent intermediate. In effect, it doesn't physically bind to the beta-lactam substrate and, therefore, is not affected by the action of beta-lactamase inhibitors, such as clavulanic acid and sulbactam.

According to Walsh, this new MBL from India is "possibly the most worrying development since Fleming gave us penicillin in 1929" (*Int J Antimicrob Agents,* 2010, S8–S14). The problem is that there are no drugs around to combat NDM-1, "Over the last 15 years, both industry and academia have flirted with designing MBL inhibitors, yet such flirtations have not yet produced anything even remotely approaching phase 1 clinical trials. This is an immense problem as the clinical fraternity must wait at least another eight years before such a molecule materialises."

To make matters even worse, NDM-1 isn't present on a single 'superbug'. It's a

resistance gene on plasmids that seems to pass readily between different bacterial species by horizontal gene transfer. And these plasmids also carry other resistant genes!

Walsh reported the analysis of isolates from 37 cases in the UK, and 143 in India and Pakistan. All the isolates were highly resistant to many antibiotic classes, including beta-lactams, fluoroquinolones and aminoglycosides. These are the main antibiotic classes for the treatment of Gram-negative infections.

Rapid Horizontal Gene Transfer

Although most isolates remained susceptible to two antibiotics, colistin and tigecycline, this isn't such a great help since both of these drugs pose problems in themselves. Colistin belongs to the class of polymyxins. It dates back to the 1940s, but has toxic effects on the kidneys and does not penetrate well into tissues. Meanwhile, tigecycline, released in 2005, is the first of a new antibiotic class called glycylcyclines but it doesn't diffuse well through blood or the bladder, rendering it ineffective for bloodstream and urinary tract infections caused by KPC and NDM-1. Furthermore, the US Food and Drug Administration (FDA) now warns that tigecycline leads to "an unexplained increased risk of death" in some patients. Not very reassuring!

There are now reports of pan-resistant NDM-1 bacteria in India, i.e. they are resistant to all the antibiotics tested! Walsh explains how bad matters could become. He points to common urinary tract infections that affect millions of people every year. "Imagine a woman going to see a medical doctor with cystitis." With no reason to suspect resistance, the doctor would prescribe antibiotic drugs that no longer work, while the infection spread unimpeded up her urinary tract, into her kidneys and, fatally, her blood. "There would be nothing to treat her with," he says.

And NDM-1 has spread fast since its first characterisation in 2008, apparently due to the speed, with which plasmids are passing into other bacteria. All the Indian isolates he reported in 2010 carried the NDM-1 gene exclusively on plasmids. Plasmids from 44 non-clonal isolates in Chennai ranged in size from 50 kb to 350 kb, whereas most of the 26 isolates from Haryana were clonal *K. pneumoniae* with plasmids sized either 118 kb or 50 kb. The UK isolates had a more diverse range of plasmid sizes, from 80 kb to > 500 kb.

Medical tourism

Meanwhile, far from thanking Walsh, the Indian Health Ministry disputed the conclusions of his 2010 *Lancet* article because Walsh had taken the opportunity to warn against practices encouraging the spread of NDM-1. In particular, the tendency for 'medical tourism': many of the UK NDM-1 cases had travelled to India to have surgical treatments, including cosmetic surgery. If other Europeans and Americans go to India for cosmetic surgery, then NDM-1 "will likely spread worldwide". Walsh was also appalled by "calls in the popular press for UK patients to opt for corrective surgery in India with the aim of saving the National Health Service (NHS) money", since such a proposal might "ultimately cost the NHS substantially more"!

Indian health officials immediately cried foul, insisting that Indian hospitals were perfectly safe for treatment. They accused Western medical doctors of enviously trying to undermine the subcontinent's booming medical tourism industry. Indian politicians described linking the new drug resistance gene to India as "malicious propaganda", blaming multinational corporations for "selective malignancy".

Bowing to the pressure, *The Lancet*'s editor apologised to the *Times of India* (11/01/11). He admitted that naming NDM-1 after India's capital city had been a "big mistake" because it "unnecessarily stigmatised a single country and city".

However, Walsh didn't seem particularly repentant about associating India with the new carbapenemase. Okay, he noted, "Not all patients infected with NDM-1-positive bacteria have a history of hospital admission in India." Nevertheless, since we all "know" that other extended-spectrum β-lactamases are "circulating in the Indian community", then perhaps we ought to be looking at the "prevalence of the NDM-1 gene in drinking water and seepage samples in New Delhi".

He published his results in April 2011: "Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environ-

Antibiotics – How they Work

More than 150 antibiotics belonging to at least 17 different classes are now available. Each antibiotic operates at a specific site within the bacterial cell. Some inhibit cell wall synthesis, notably, the beta-lactams (penicillins, cephalosporins, carbapenems, monobactams), glycopeptides and cyclic lipopeptides (daptomycin). Others disorganise cell membranes (e.g. polymyxins). They can inhibit synthesis of proteins (e.g. aminoglycosides, chloramphenicol and tetracycline), DNA (fluoroquinolones) and RNA (rifamycins) or target particular biochemical pathways, such as folic acid synthesis (e.g. methotrexate and sulfonamides). And there are other mechanisms, e.g. metronidazole cross-links to cysteines on enzymes.

Bacteria – How they Resist

Bacteria have developed resistance mechanisms against all antibiotic classes. The four main mechanisms are:

- Inactivation or modification of the antibiotic molecule, e.g. cleavage of the beta-lactam ring in penicillins by beta-lactamases and carbapenemases.
- Alteration of the target site, e.g. the elimination or reduction of antibiotic binding seen with erythromycin and lincomycin resistance; or the overproduction of the antibiotic target (titration) found with sulfonamides and trimethoprim.
- Bypassing an inhibited reaction by alteration of metabolic pathways, e.g. some bacteria can resist sulfonamide inhibition of folic acid synthesis by using preformed folic acid instead of the precursor molecule.
- Reduced drug accumulation: by decreasing drug permeability into the cell (e.g. for chloramphenicol) and/or increasing active efflux, pumping the drugs back out (e.g. tetracycline).

Bacteria also seem to have found an economic mechanism – they've targeted the profitability of antibiotic development by drug companies. A pre-emptive strike! Just the prospect that bacteria will rapidly inactivate any new drug is now sufficient to discourage pharmaceutical companies from joining the battle.

mental point prevalence study" (*Lancet Infect Dis,* 2011; 11: 355–62). To get the environmental samples for lab analysis, Walsh took the unusual step of recruiting a television news crew from the UK's Channel 4.

Between 26/09 and 10/10/2010, they collected the swabs (and photographic evidence). Some 51 of 171 seepage samples and 2 of the 50 drinking water samples were NDM-1 positive. As a control, or possibly to demonstrate the comparative cleanliness of the Welsh capital, Walsh took 70 sewage effluent samples from Cardiff – these were all NDM-1 negative.

The conclusion: "Oral–faecal transmission of bacteria is a problem worldwide but its potential risk varies with the standards of sanitation. In India, this transmission presents a serious problem". However, Walsh stressed that his study showed the need for an international surveillance of resistance. And he insisted that he would be "delighted" to advise and help scientists, clinicians and government officials from the Indian sub-continent in order to make progress.

Natural selection pressures

What factors favour the rise of antibiotic resistance in bacteria? The classic idea is that when sensitive bacterial populations are exposed to an antibiotic, they will all die except for a few bacteria that have some phenotype that permits survival. These bacteria continue to grow and each time they are confronted with the antibiotic, selection pressure favours them. With each new generation, the bacteria carrying the resistant gene become more and more dominant in the population until the drug is completely ineffective.

Most people carry more than 500 different bacterial species in their guts but 10 species predominate. The human body has 10^{13} cells but inside us there are around 10^{14} bacterial cells, accounting for 1-2.5kg of our body weight. Human skin also carries several hundred species. With such huge numbers of fast-growing bacteria, any antibiotic resistant genes that appear in response to selection pressure could become established and continue to spread. It's just a question of time. However, some medical and agricultural practices are accelerating this selection.

Medical selection pressures

For a start, antibiotic resistance is facilitated by their misuse as medicine. Taking antibiotics for too short or too long a period, at too low a dosage, at substandard potency or for the wrong disease probably improves the chances of survival for moderately resistant bacteria. The WHO claim that up to half of bacterial pneumonia cases are not treated with appropriate antibiotics. Meanwhile, there is still a tendency to give people antibiotics to treat viral infections.

Hospitalised patients constitute one of the main reservoirs of antibiotic-resistant microorganisms. And patients with resistant bacterial infections are in close proximity with other patients whose vulnerable state makes them susceptible to acquiring such nosocomial (hospital) infections. Health professionals could help reduce the spread of infection in health care facilities by ensuring good hygiene, washing their hands better and more frequently, taking more care to maintain aseptic practices, sterilising and disinfecting surfaces and equipment, improving waste management, etc. However, the scale of many of these problems varies enormously from country to country.

India has enormous problems of poverty and infrastructure. Walsh's study of NDM-1 highlighted the problems of inadequate sewage treatment. A direct result is that untreated faecal matter is contaminating water supplies. These are used to irrigate food plants and as drinking water. Another major headache is that antibiotics are freely available from pharmacies without medical prescription. This means that anyone who feels sick with an upset stomach or diarrhea can directly buy whatever antibiotic treatment is available, whether it is appropriate or not. Needless to say, the potential for the selection and rapid spread of antibiotic resistance genes is far greater than in Europe and North America. Or is it?

It might seem incredible in the light of the growing antibiotic resistance crisis but most antibiotics used in the US and UK are given to animals, and are not even for therapeutic purposes! In 2001, the Union of Concerned Scientists estimated that greater than 70% of the antibiotics used in the US are given to food animals (for example, chickens, pigs and cattle) in the absence of disease. Antibiotics at low doses promote growth in farm animals (albeit by a few percent) – more meat in less time!

Agricultural selection pressures

Already in the 1990s, the problem of adding antibiotics to animal feed was recognised. George Khachatourians wrote in 1998 about the agricultural use of antibiotics, linking widespread use of antibiotics in animals to the development of antibiotic resistance in human flora (*CMAJ,* 159:1129-36).

For example, the multidrug-resistant *Salmonella typhimurium* definitive type 104 (DT 104) initially emerged in UK cattle in 1988; it was subsequently found in meat and meat products from other domestic animals, as well as unpasteurised milk from other locations. Human illness occurred through contact with farm animals and consumption of beef, pork sausages and chickens. Similarly, vancomycin-resistant enterococci (VRE) have links to agricultural practice: since the 1950s, vancomycin has been in medical use but since the 1980s it has also been given to farm animals. VRE was first isolated from sewage treatment plants in Britain and small towns in Germany and later in manure samples from pig and poultry farms. It has since been transmitted to humans through the food chain in Germany, Norway and the Netherlands.

There have even been some antibiotics 'reserved' for animal use only. Unfortunate-

ly, structural similarities to human antibiotics have meant that resistance to the former has impacted on the latter. For example, turkeys given virginiamycin harboured bacteria resistant to the structurally similar antibiotics: streptogramin, quinupristin and dalfopristin. Animal feed supplemented with tylosin led to the development of erythromycin-resistant Streptococci and Staphylococci in the animals *and* their caretakers.

In 1998, EU health ministers voted to ban four antibiotics widely used to promote animal growth (despite contrary scientific advice). Regulations banning the use of antibiotics in animal feed became effective in 2006. In the USA, most legislation to ban antibiotics in feed has so far been successfully blocked by the food, animal and pharmaceutical industries. However, this EU ban is only for antibiotics as growth promoters – farmers can continue to give animals drugs for therapeutic treatment and as a precautionary measure to prevent the spread of disease.

"Death wish: Routine use of vital antibiotics on farms threatens human health" (*The Independent*, 17/06/11) reports how antibiotic use on British farms has risen dramatically in the past decade. Use of three classes of antibiotics rated as "critically important in human medicine" by the WHO – the cephalosporins, fluouroquinolones and macrolides – has increased "up to eightfold in the animal population over the past decade". Over the same period, livestock numbers have fallen: by 27% in the case of pigs, 10% for cattle and 11% for poultry.

Shortage of new antibiotics

In May 2011, a new type of cephalosporin-resistant MRSA was identified in British cow's milk. Cephalosporins are routinely used in cows to prevent udder infections, which are mostly caused by *S. aureus* and arise more frequently due to intensive milking. Although pasteurisation kills MRSA in milk, past experience has shown it spreads directly from cattle to farm workers and on into the community.

Richard Young, from the Soil Association, says the increasing medicinal use of antibiotics is driven by the unnatural demands of intensive farming, "The basic problem is that supermarkets see animals as cogs in a big industrial process. The profit margins are incredibly tight. Most of these problems can be avoided by having less intensive systems so the animals are naturally healthier."

Between 1998 and 2008, the FDA approved 13 new antibiotics. Only three had new mechanisms of action. In 2009, the Infectious Diseases Society of America found only 16 antibiotics at any stage of development – eight to treat Gram-negative bacteria, but none could be used against highly resistant Gram-negatives, such as KPC and NDM-1 bacteria.

Walsh points at these statistics, "Without explicitly saying so, most of the pharmaceutical industry has decided that drugs to treat carbapenem-resistant infections are so challenging to develop and can be used for so short a period before resistance arises, that they are not worth research and development time."

Unlike other classes of drugs, antibiotics are distinctive in that their use precipitates their obsolescence by selecting for resistant microbes – they start losing market value as soon as they are used. For economic reasons, new antibiotic discovery has become a low priority for the pharmaceutical industry, despite the growing clinical need. Alanis from Lilly Research explains the internal pressure within pharmaceutical companies whose R&D budgets are allocated by Therapeutic Area. Companies with a Division of Infectious Diseases have a limited budget to develop "anti-infectives". But in recent years, antiretroviral agents have been seen as more potentially profitable products (e.g. to treat HIV). This has resulted in directing the R&D budget towards developing antiretroviral drugs and away from other antimicrobials, especially antibiotics.

Antibacterial research productivity has also been much lower than expected. Alanis notes that new technologies such as genomic research, combinatorial chemistry and high throughput screening, once heralded as capable of yielding a large number of novel targets amenable to modulation via their interaction with new drugs, have "failed to deliver on their promise". "No new antibiotic classes have been discovered through the use of these new techniques."

Ironically, measures to control the spread of antibiotic resistance, such as not prescribing antibiotics unnecessarily, have also reduced commercial demand with a "net negative effect on the economic incentive to invest in the creation of newer antibiotics".

The WHO have called for "push" and "pull" incentives to encourage antibiotic R&D. Basically, this means using public money to subsidise the costs of research and clinical trials to develop new drugs, while providing guarantees that pharmaceutical companies won't lose money when they finally market the drugs. Along these lines, the US Senate has recently introduced the Generating Antibiotic Incentives Now (GAIN) Act, to "spur development of new antibiotics to combat the spread of antibiotic resistant bacteria".

JEREMY GARWOOD

An accompanying article at *www.labtimes.org* looks at recent research on the 'Environmental Antibiotic Resistome', which has found that soil bacteria are a major natural source of antibiotic resistant genes.

ONE FINE DAY IN THE LAB...

BY LEONID SCHNEIDER